Investigating T-cell interactions towards cultivation requirements

(Untersuchung von T-Zellinteraktionen hinsichtlich der Anforderungen an die Kulturbedingungen)

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Abstract (English)

The 24-well plate culture system is widely used to culture antigen specific T-lymphocytes which are in turn used for immunotherapeutic applications with success [1-3]. However, this system has drawbacks when used for "large-scale" T-cell generation, for example regarding cross-contamination and bio-safety, it is material-and labour-intensive, and costly under GMP conditions and ultimately, there is a higher risk of human error in the implementation. Thus there is a pressing need to develop a new culture system for the large-scale T-cell generation. Studies show that T-cells require special culture conditions that have to be maintained to ensure proper growth and functionality in order for them to be usable in immunotherapeutic applications [4-6]. They also rely heavily on cell to cell interaction for their maturation and growth [7]. Physical forces applied to T-cells during culture have also been shown to influence T-cell growth [8-10] but it is still unknown if the culture system itself can influence their growth. This information is important when designing and building a new culture system for T-cells different to the 24-well plate culture system which is now a standard for everyday culture of T-cells.

In this project we investigate the physical interactions between T-cells and culture system in the view to develop a new closed culture system to grow antigen specific T lymphocytes. We look at interactions that have the highest probability of influencing T-cell growth and they are as follows: T-cell ↔ material surface interactions, T-cell ↔ culture chamber interactions, T-cell ↔ observer interaction, T-cell manipulation during inoculation, culture and harvesting and finally a study on Multi-factorial influence on T-cell growth.

The T-cell ↔ material surface interactions were found to have the strongest influence on T-cell growth while most of the materials we tested were compatible except for Tecoflex which showed the worst growth. The next strongest influence is the T-cell ↔ culture chamber interactions where surface roughness and culture chamber geometry seem to favour T-cell growth during different time points of the culture period. The growth was also affected by non-continuous surfaces (such as electrospun fibres) where continuous surfaces proved to be much superior. Human judgement of the culture conditions can also influence T-cell growth and we tried to minimize this by looking at T-cell ↔ observer interactions and developed a fixed splitting schedule for culturing T-cells. The manipulation of T-cells during the stages of inoculation, culture and harvesting is also important especially in a closed system and we found that mixing tables can be safely used to manipulate and homogenize the medium during these stages. Finally we also found that the factors we investigated can have a multi-factorial influence on T-cell growth depending on the way the culture system is designed as seen in the two bioreactors we compared in our last experiment.

It is now clear that both the culture system and culture process strongly influence T-cell growth and the parameters we have investigated need to be followed when designing a culture system for T-cells. Additionally, we need to keep in mind the multi-factorial influence on T-cell growth which can occur when all of the factors are acting together, which might have a different effect on T-cell growth. This study also showed us that there is still a lot we do not know about T-cell culture and further work done in this field might reveal other new influences to T-cell growth during culture.

Abstrakt (Deutsch)

Zur in vitro Kultivierung von Antigen-spezifischen T Lymphozyten, welche erfolgreich in immuntherapeutischen Ansätzen verwendet werden, ist das 24-Loch-Platten Kultivierungssystem weit verbreitet. Allerdings hat dieses System Nachteile bei der „large-scale“ T-Zellgeneration, bezüglich Kreuzkontamination und Sicherheit. Darüber hinaus ist es material- und arbeitsaufwändig und kostenintensiv unter GMP Bedingungen. Letztlich besteht ein höheres Risiko für Fehler bei der Durchführung. Deshalb ist die Entwicklung eines neuen Kultursystems für die T-Zellgeneration im großen Maßstab nötig. Studien zeigen, dass diese T Zellen spezielle Kulturbedingungen benötigen, die Wachstum und Funktionalität gewährleisten, um sie im Anschluss für immuntherapeutische Anwendungen nutzen zu können. Für Reifung und Wachstum sind T Zellen außerdem stark auf Zell-Zellinteraktionen angewiesen. Zusätzlich wurde gezeigt, dass physikalische Kräfte, die während der Kultivierung auf die T Zellen wirken, einen Einfluß auf das Zellwachstum haben. Es ist allerdings nicht bekannt, ob das Kultivierungssystem selbst einen Einfluss auf das T Zellwachstum und die Funktionalität hat. Diese Information ist wichtig für das Design eines neuen Kultivierungssystem, das sich vom routinemäßig verwendeten Standard 24-Loch-Platten System unterscheidet.

In diesem Projekt soll die physikalische Interaktion zwischen T Zellen und Kultursystem untersucht werden, mit dem Ziel, ein neues geschlossenes Kultivierungssystem für Antigen-spezifische T Zellen zu entwickeln. Wir untersuchten folgende Faktoren, die eine hohe Wahrscheinlichkeit haben, das T Zellwachstum zu beeinflussen: 1.) Interaktionen von T Zellen abhängig vom Oberflächenmaterial sowie der Kulturkammer, 2.) die Abhängigkeit vom Betrachter, 3.) die Abhängigkeit von der Manipulation während der Inokulation, der Kultivierung und Ernte der T Zellen, sowie 4.) den multifaktoriellen Einflüssen auf das T Zellwachstum.

Die Interaktion zwischen T-Zellen und dem Material hat den größten Einfluss auf das T-Zellwachstum, wobei die meisten von uns getesteten Materialien kompatibel waren. Eine Ausnahme

stellte Tecoflex dar, welches das schlechteste T-Zellwachstum zeigte. Den nächstgrößten Einfluss hat die Interaktion zwischen T Zellen und Kulturkammer, wobei eine raue Oberflächenstruktur und die Geometrie der Kulturkammer unterstützend auf das T-Zellwachstum zu verschiedenen Zeitpunkten der Kulturperiode wirkt. Das Wachstum wurde von diskontinuierlichen Oberflächen negativ beeinflusst (wie z.B. Fasern), kontinuierliche Oberflächen erwiesen sich als überlegen. Die Beurteilung der Kulturbedingungen durch den Beobachter können das T-Zellwachstum ebenfalls beeinflussen. Durch die Untersuchung des T-Zell-Beobachtereffekts und der Entwicklung eines fixen Weiterkultivierungsplans wurde versucht, diesen Faktor zu minimieren. Besonders in einem geschlossenen System ist die Manipulation der T-Zellen während der Inokulation, Kultur und Ernte von Bedeutung. Hierzu wurde gefunden, dass zur Manipulation und Homogenisierung des Mediums Schütteltische sicher verwendet werden können. Schließlich können die untersuchten Faktoren einen multifaktoriellen Einfluss auf das T-Zellwachstum haben, wie bei den beiden zuletzt untersuchten Bioreaktoren abhängig vom Design des Kultursystems gezeigt wurde.

Aus den hier gezeigten Untersuchungen geht hervor, dass das Kultursystem und der Kultivierungsprozess das T-Zellwachstum stark beeinflusst. Die untersuchten Parameter müssen beim Design eines T-Zellkultursystems beachtet werden. Zusätzlich, müssen die multifaktoriellen Einflüsse auf das T-Zellwachstum beachtet werden, die beim Zusammenspiel der unterschiedlichen Faktoren einen anderen Effekt auf das T-Zellwachstum zeigen können (als einzelne Faktoren). Diese Studie zeigte auch, dass es noch viele unbekannte Größen in der T-Zellkultur gibt und dass weiterführende Arbeiten in diesem Feld neue Einflüsse auf T-Zellwachstum in Kultur aufdecken können.

Contents

Abbreviations

Acronym	Definition
AICD	Activation Induced Cell Death
APC	Antigen Presenting Cell
APC	Allophycocyanin
BFA	Brefeldine-A
BLZF	Basic Leucine Zipper Nuclear Factor
CD	Cluster of Differentiation
CM	Central Memory
CMV	Cytomegalovirus
DMSO	Dimethyl Sulfoxide
EBNA	Epstein-Barr Nuclear Antigens
EBV	Epstein-Barr virus
ECD	Texas Red
EM	Effector Memory
EP	Electrospun Parallel aligned
ER	Electrospun Random aligned
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GMP	Good Manufacturing Practice
HIV	Human immunodeficiency virus
HZG	Helmholtz-Zentrum Geesthacht
IFN	Interferon Gamma
IL	Interleukin
IONO	Ionomycin
IU	International Unit
LD	Live Dead stain
LMP	Latent Membrane Protein
MACS	Magnetic-Activated Cell Sorting
MHC	Major Histocompatibility Complex
MS	Magnetic Separator
NCBI	National Center for Biotechnology Information
NKT	Natural Killer T-cell
NO	Nitric Oxide
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PC	Polycarbonate
PE	Phycoerythrin
PEI	Poly(ether imide)
PEU	Poly(etherurethane)
PMA	phorbol 12-myristate 13-acetate
PS	Polystyrene
PSAN	Poly(styrene-co-acrylonitrile)
RPMI	Roswell Park Memorial Institute medium
RWV	rotating wall vessel (bioreactor)
SEM	Scanning Electron Microscope
SPSS	Statistical Package for the Social Sciences
TCR	T-Cell Receptor
TFX	Poly(etherurethane) or Tecoflex®

Introduction

T lymphocytes and Immunotherapy

The T lymphocyte is a special type of white blood cell that is part of the adaptive immune system. They react only to specific antigens through a special T-cell Receptor (TCR) and are responsible for cell-mediated immunity in humans. T-cells develop in the thymus and mature upon reaching secondary lymphoid tissue where they encounter antigen presenting cells (APCs) which are responsible for their activation. Meanwhile, they also develop memory T-cells that either circulate in the blood (known as effector memory T-cells) or remain inside secondary lymphoid tissue (known as central memory T-cells). These memory T-cells can remember a pathogen from previous infections making them optimal for fighting recurrent infections by the same pathogen.

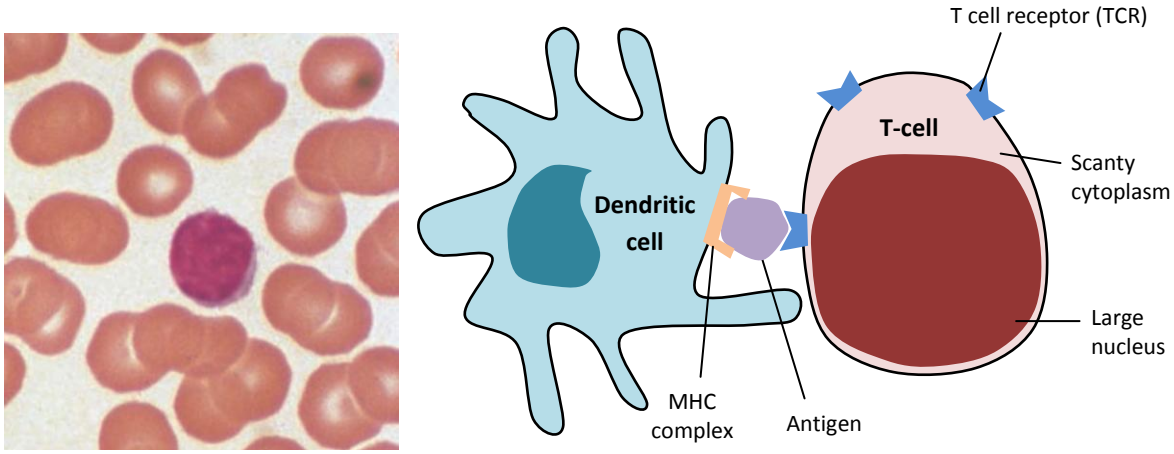


Figure 4.1: Lymphocyte surrounded by red blood cells and antigen recognition via TCR.
(Left) *A lymphocyte surrounded by red blood cells. Notice the large nucleus surrounded by scanty cytoplasm. Taken from Janeway's immunobiology 8th edition, Kenneth Murphy.* ***(Right)*** *T lymphocyte recognizing antigen via TCR. The Dendritic cell is also known as an antigen presenting cell (APC).*

Once activated by an APC; memory T-cells are capable of rapidly proliferating and producing more memory cells along with effector cells that are cytotoxic. These effector T-cells are capable of seeking and identifying infected cells via the TCR. Hence, when a cytotoxic T-cell recognizes peptides presented on the MHC-I receptor (which is present on all nucleated cells in the body), they kill the infected cell by releasing cytotoxins and signal for recruitments from the immune system. This form of immunity is very useful to control opportunistic viral infections such as Epstein-Barr virus (EBV) and Cytomegalovirus (CMV); as these viruses are known to invade an organism and remain inside the host cell to avoid detection by the innate immune system [11-13]. Hence the main aim of T lymphocytes is to seek and destroy pathogens that are hiding inside host cells and in the process help in complete eradication of an infection from the body.

When the immune system is weakened (e.g. after immunosuppressive drugs or diseases like HIV or individuals with weak immune systems such as the elderly and very young) there is a chance for opportunistic infection by viruses mentioned earlier. These are life-threatening and have become a major cause of concern especially in post-transplant patients [3, 14]. In order to combat such infections, research has shown that T lymphocytes generated from the patient's own blood can be used to fight these infections [1-3]. This is done by first extracting T-cells specific to the invading pathogen and then culturing them in-vitro until sufficiently high cell numbers are reached. These cells are then re-introduced to the patient to treat the underlying infection or as prophylactic treatment from opportunistic infection. This form of treatment is known as immunotherapy and shows promising results with immunocompromised patients [3, 14].

The 24-well plate culture system for antigen specific T lymphocytes

In this project we focused on the culture of EBV specific T-cells which are used for immunotherapeutic applications. In a laboratory T lymphocyte culture is normally carried out in the 24-well plate culture system which works very well with good results. It is a known standard for culturing T-cells and results are reproducible.

If we examine this culture system, as in the schematic (figure 4.2), a typical plate is made up of 24 wells all of which have a fixed shape (circular), fixed dimensions (width, breadth and height). The plates are generally made of surface treated polystyrene (oxygen plasma treated) which is a known biocompatible material. The plate has a cover that sits on top and can be considered an **open culture system** since direct access to the culture contents is possible by the observer.

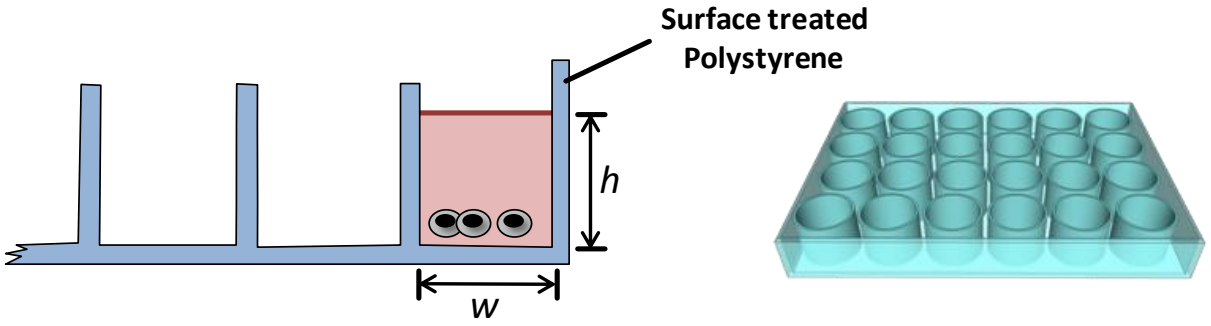


Figure 4.2: Schematic of a typical 24-well plate.

During culture, complete medium is used and maintained at a volume of around 2ml per well throughout the culture period. Once T-cells reach confluence in a single well, the cell clumps are

broken and re-suspended into two wells along with fresh medium to maintain medium height (figure 4.3a) and this is repeated as and when required depending on the rate of T-cell growth which is donor dependant (figure 4.3b). The medium colour changes due to accumulation of cellular metabolites which also gets replaced with fresh medium when required.

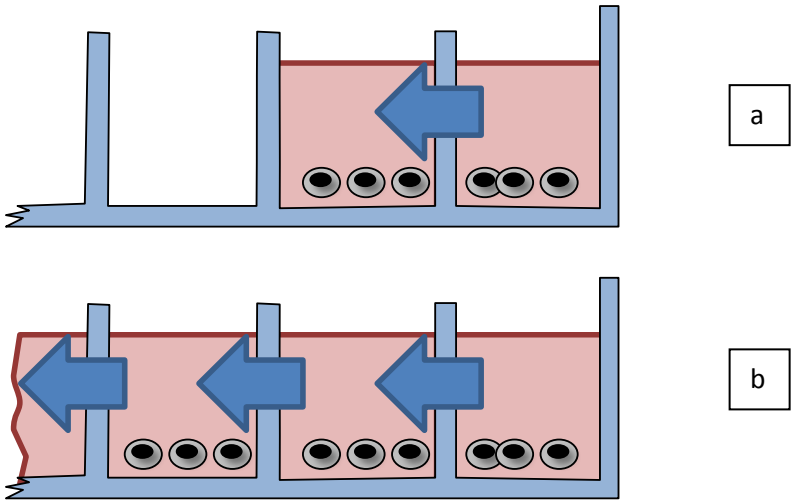


Figure 4.3: Expansion method of T cell culture by splitting.

The entire culture process described so far is regularly employed by scientists working with T-cell cultures and very few deviations are made to this method of culture. However, this brings up the question: if we were to design a new culture system for T-cells (such as a bioreactor); would the new system influence T-cell growth differently? It is an important point to address since these T-cells are later used in immunotherapy and any influence to its growth during culture would be undesirable.

The problem

There are certain characteristics that we expect in T-cells (in terms of functionality, phenotype and cell number) when they are grown in 24-well plate culture systems. These same characteristics are well known and indicate how well T-cells would respond in immunotherapeutic applications [15-18]. Hence, any changes to these characteristics would make the T-cells unsuitable for use in immunotherapy. However, very less information is currently available on how a culture system physically interacts with T-cells and influences their functionality, cell number and phenotype.

Studies have previously been done about culture conditions that are “desirable” in our culture systems [4-6]. T lymphocytes are different from most cells grown in culture as they tend to be semi-adherent and disperse easily when there is turbulence in the medium. Early studies have shown they

have unique requirements in terms of gas supply, pH, temperature, osmolality and cytokine supply[4-6]. The cell proliferation rates vary between blood donors and they also tend to behave differently to physical influences during culture [8-10]. Therefore, when constructing a closed culture system (like a bioreactor) for culturing antigen specific T-cells; information about how the culture system physically interacts with T-cell growth is vital for its design and construction. Any negative influences on T-cell function; phenotype or cell number would make the system unacceptable for use. Additionally, the system must permit harvest of T-cells without the need to apply chemicals or excessive force. This will minimize the damage to T-cells maintaining their functionality and viability.

We decided to focus on the main interactions T-cells have with the culture system and a logical list of possible major interactions responsible for T-cell growth was decided as follows:

1. T-cell ↔ material surface interactions
2. T-cell ↔ culture chamber interactions
3. T-cell ↔ observer interaction (splitting technique during culture)
4. T-cell manipulation during Inoculation, culture and harvesting
5. Multi-factorial influence on T-cell growth

Please see next page for the Experimental design which illustrates how we decided to proceed with our investigations.

The reason for focusing on closed culture systems in our study rather than an open one is because the 24-well plate culture system has major disadvantages when used for large-scale T-cell generation. Usually various patients' T-cells would be grown in the same lab and probably the same incubators using 24-well plates which are open systems. This generates a pressing need to prevent cross-contamination between T-cells from different patients. Additionally, 24-well plates tend to increase in number as the cells keep growing making it harder to store several patient T-cells in a single place. It is material and labour intensive, since a large number of plates are needed per patient, making its use in the GMP very expensive and complicated. This also includes a higher risk of human error as a result of the complicated nature of the system. Hence, a closed system would be the best solution logistically, economically and sterility wise for the large scale generation of T-cells from various patients. An additional requirement is that the system should be able to contain the entire culture without the need for separate vessels as T-cells grow and expand while solving most of the above mentioned disadvantages.

The aim

The aim of this PhD project is to investigate the interactions between T-cells and the culture system which may help in the design of a closed culture system for antigen specific T-cells. The 24-well plate culture system is a gold standard for comparison in our studies and has been used in all our experiments whenever possible.

Experimental design:

In order to understand how the interactions between T-cells and their culture system affect T-cell function, phenotype and maturation, the following plan of approach was decided (based on mechanical aspects of the culture process).

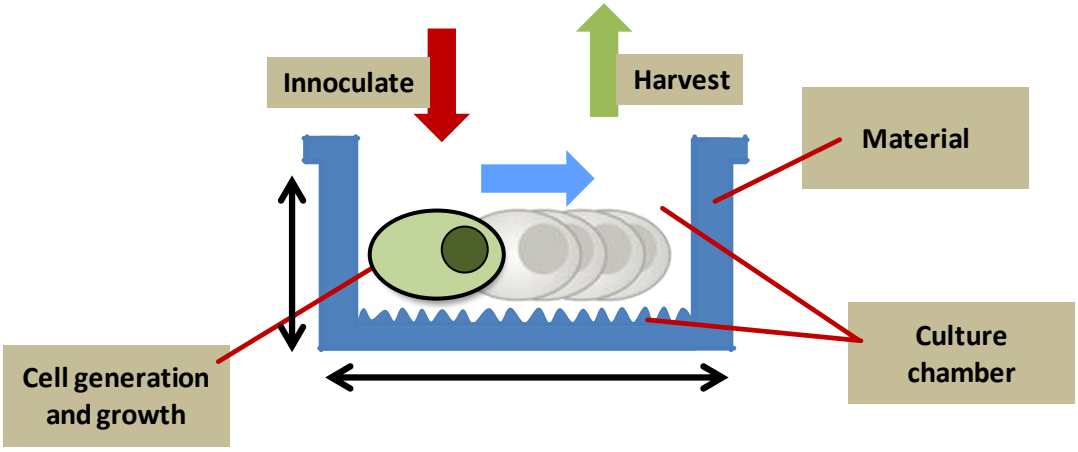


Figure 4.4: T cell interactions (during culture).

These comprise interactions that we believe could influence the quality of T-cells generated in a culture system and also may be important when designing a bioreactor for T-cell culture in future.

1. **Material of culture system**

   The chemistry of materials used in construction could have a significant influence as T cells are prone to settle down to the bottom surface of the culture system in the absence of turbulence in the medium.

2. **Culture Chamber**

   The shape and dimensions of the culture chamber along with the mechanical characteristics of the "culture surface" is probably the next important factor of influence on T-cell growth. Changes to any of these parameters would adversely influence cell to cell interaction and

cytokine availability which in turn might influence cell number and maturation of T-cells (as discussed later).

3. Cell generation and growth by observer

The manipulation of T-cells by the observer during expansion might affect the final number of viable cells at the end of culture and hence could also be a determining factor in the production process.

4. Manipulation during Inoculation, culture and Harvest

Evidently the generation of T-cells for immunotherapy has to be carried out in sterile conditions and GMP standards. This demands that the inoculation, culture and harvest of T-cells in the culture system should be executed in a sterile fashion without the possibility of contamination. A closed system would solve this but the problem remains in the manipulation of T-cells in such a system since direct manipulation is not possible in a closed system. Also, the removal of T-cells during harvest from the closed system should be efficient, sterile and done in a manner so that T-cells are not physically damaged during the process.

5. Multi-factorial influence on T-cell growth

Finally two existing bioreactors were also used to investigate how T-cell growth is affected by multi-factorial influences.

Table 4.1: Experiments done to investigate T cell interactions.

T-cell interactions	Experiments
Materials	Material testing (R00) –PS,PEI,PEU,PSAN,PC
	Material testing (R30) –PS,PEI,PEU,PSAN,PC
	Material testing (R45) –PS,PEI,PEU,PSAN,PC
	Material induced T-cell function experiment
Culture chamber (shape, size, volume and culture surface)	Geometry testing
	Roughness testing experiments (R00, R30 , R45)
	Electro-spun materials
T-cell expansion technique	Cell number experiments
	Fixed splitting experiments
	Peptide pool titration experiments
Manipulation during Inoculation, culture and harvesting	Cell culture dissociation experiments
Comparison of existing systems [1]	System comparison experiments

[1] *In addition two existing systems were also investigated to find out additional influences to T-cell growth which is unique to such systems.*

Results

The following results are divided into sections and are ordered in a chronological manner, hence the first set of experiments have lead to further experiments in subsequent sections where indicated.

- The factors investigated in this study all have been found to influence T-cell growth in one way or the other. However, when combined together they might have an influence on T-cell growth and function which is different from their individual influences.

- Thus it is necessary to consider the combined influence of the factors discussed in this study when constructing a culture system for antigen specific T lymphocytes.

Section 1:

T-cell material interactions

T-cell material interactions

In order to investigate if T-cells can be influenced by the culture chamber material (floor and walls), a set of material testing experiments were done using material cup inserts in 24 well plates (see figures 5.1 and 5.2 below and pages 69 and 113 for details about the advantages of using material cups instead of material fragments).

The T-cells were isolated from fresh blood (healthy donors) using the pan T-cell stimulation protocol (please see page 107) and the cells were placed in approaches as in figure 5.2 below. After culture for 8 days the cells were harvested and counted. In this experiment the use of 24-well plate was just for comparison and cannot be used as a control due to various differences such as: size of culture chamber, oxygen plasma surface treatment and the higher volume of medium available to the cells growing in a 24-well plate culture compared to the cups (more of this in the discussion section).

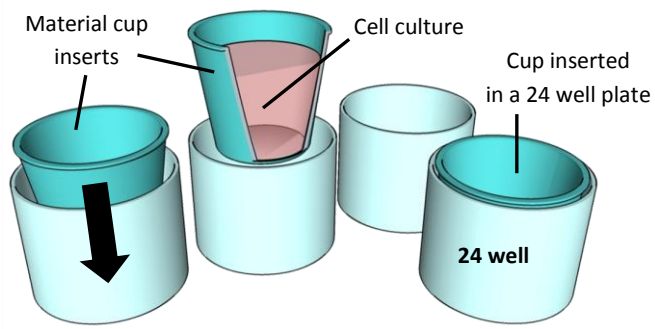


Figure 5.1: Material cup placement in 24 well plates.

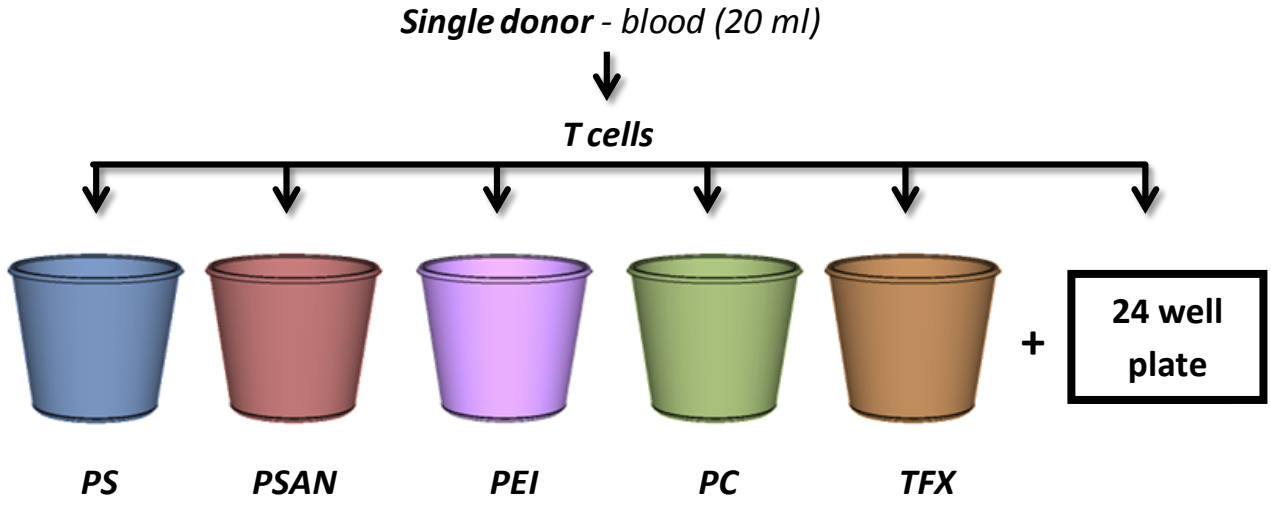


Figure 5.2: Material testing experiment using material cups with roughness R00. *Fresh blood was taken from healthy donors (20 ml) and T-cells generated with the pan T-cell stimulation protocol. The cells were plated in 5 types of materials along with a normal 24-well plate for comparison. PS = Polystyrene, PSAN = Poly(styrene-co-acrylonitrile), PEI = Poly(ether imide), PC = Polycarbonate, TFX = Poly(etherurethane) or Tecoflex®*

1. Material testing using smooth cups (R00):

The five materials used here were all of the smooth variety (R00) (please see: "*material cups and roughness"* in "*methods*" page 113). In this experiment six different donors were used (ages 28 to 35) and all of them have no known health related problems.

The following is the result obtained after 8 days of culture in 5 different material cup inserts and 24-well plate:

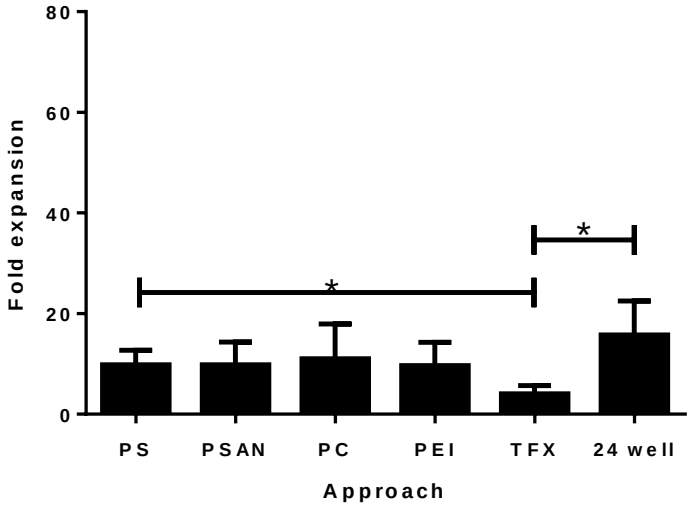


Figure 5.3: T-cell fold expansion on 5 materials after pan T-cell stimulation and 8 days of culture.
*T-cells generated from fresh blood were cultured on five material cup inserts with smooth culture surface (R00) for 8 days (see fig 5.2). The 24-well plate culture was used for comparison. Fold expansion was calculated at the end of culture. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate and TFX = Tecoflex®. Statistical analysis was performed using Wilcoxon test. n = 6 (this experiment repeated 6 times using 6 different healthy donors); *p<0.01*

Tecoflex (TFX) demonstrates the lowest fold expansion (implying a negative effect on T-cell growth) out of all the materials tested and quite significantly compared to the 24-well plate culture and polystyrene (PS) material. Please see figure 5.9, page 26, regarding the activation status of T-cells when grown on TFX. The other four materials: PS, PSAN, PC and PEI have similar fold expansions indicating no affect on T-cell growth. However, what is striking in this figure is that the 24-well plate culture has a visibly higher fold expansion compared to the five materials (please see discussion for further explanation). The figure 5.4 on the next page shows the appearance of T-cell cultures on day 8 of culture before harvest.

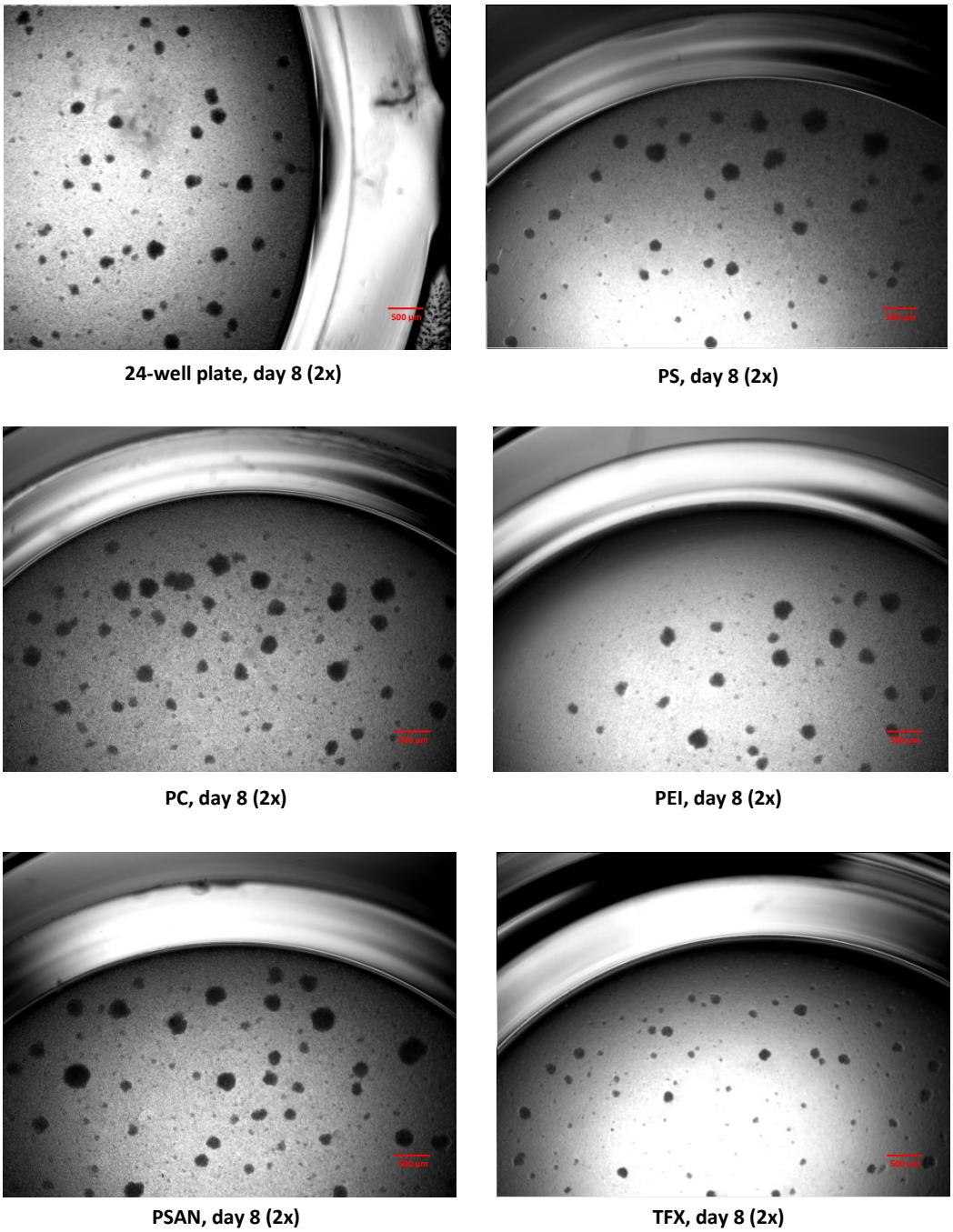


Figure 5.4: Appearance of T-cell cultures grown on R00 materials on day 8 before harvest.
T-cells generated from fresh blood were cultured on material cup inserts with smooth culture surface (R00). The 24-well plate culture was used for comparison. At the end of culture pictures were taken with a high content screening machine. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. The scale (red line) is 500µm in the six pictures.

These images were taken using a high content screening machine (Operetta system) from Perkin Elmer Inc, USA. Most notable in figure 5.4 is that the cell clumps are much smaller in TFX (lowest right) compared to the rest on day 8 before harvest.

2. Material testing using cups with roughness grade R30:

The previous experiment was repeated again with the same materials and experimental set-up but this time using roughness "R30". These are material cup inserts that have physically modified culture surfaces with a roughness grade denoted by R30. The T-cell s were isolated from fresh blood (four healthy donors) using pan T-cell stimulation protocol and after 8 days of culture on R30 materials the cells were harvested and cell numbers determined as shown in figure 5.5.

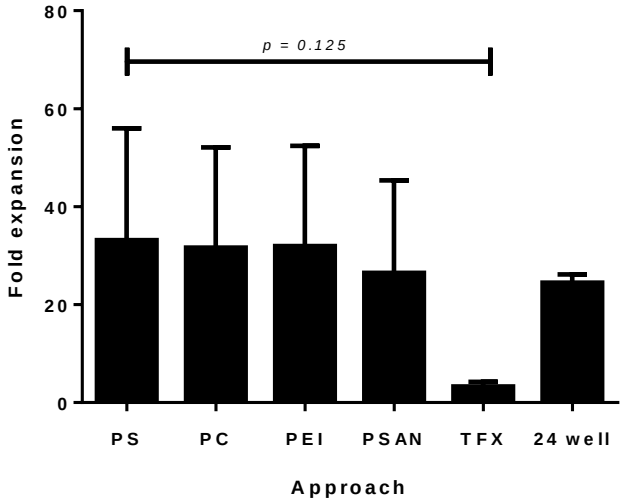


Figure 5.5: T-cell fold expansion on 5 materials with roughness R30 after 8 days of culture.
T-cells generated from fresh blood were cultured on five material cup inserts with rough surface (R30) for 8 days (see fig. 5.2). The 24-well plate culture was used for comparison. Fold expansion was calculated at the end of culture. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate and TFX = Tecoflex®. Statistical analysis was performed using Wilcoxon test, n = 4 (this experiment repeated 4 times using 4 different healthy donors).

Tecoflex (TFX) again demonstrates very poor T-cell growth while there is almost similar growth among the other four materials PS, PC, PEI and PSAN. However, what is interesting this time is that the growth in these four materials have matched the growth seen in the 24-well plate cultures (fold expansion of around 24.5) and some even exceed it by a small margin. This indicates a positive influence of rough surfaces on T-cell growth and is further examined in the roughness testing experiments (page 39). The figure 5.6 on the next page shows the appearance of T-cell cultures after growing on material inserts with roughness R30 on day 8 before harvest.

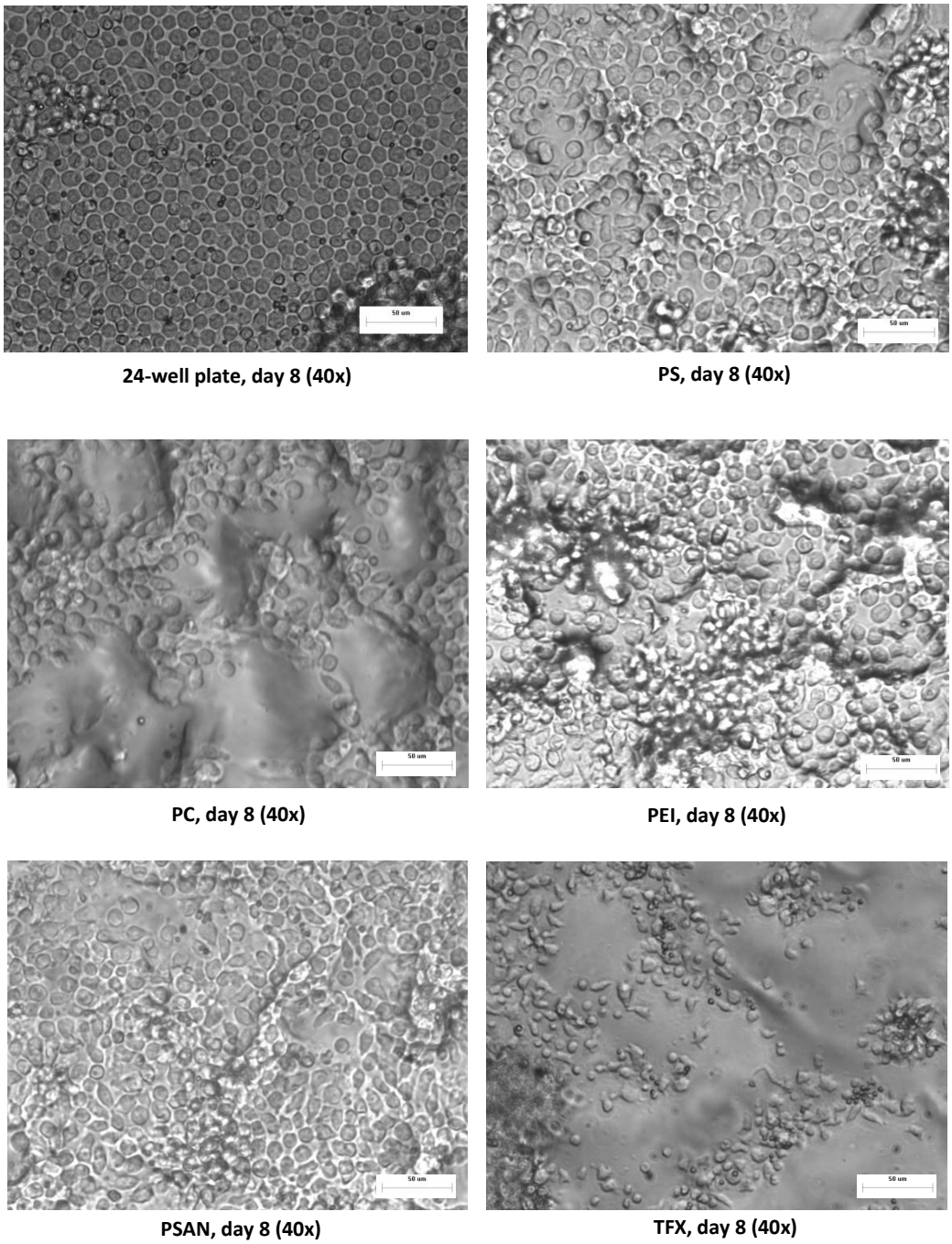


Figure 5.6: Appearance of T-cell cultures grown on R30 materials on day 8 before harvest.
T-cells generated from fresh blood were cultured on material cup inserts with rough surface (R30). The 24-well plate culture was used for comparison. At the end of culture pictures were taken with a light microscope. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. The scale is 50µm in the six pictures.

These images were taken with a light microscope (Axiovert 40CFL) from (Carl Zeiss Microscopy). The number of cells present on the surface of the material TFX appears to be much lesser (lowest right) compared to other materials on day 8 of culture before harvest.

3. Material testing using cups with roughness grade R45:

Then the same experiment was repeated again with the same set-up as before but this time using material cup inserts with a roughness grade "R45". T lymphocytes were isolated from fresh blood using the pan T-cell stimulation protocol and after 8 days of culture in material cup inserts with roughness R45, the cells were harvested and cell numbers counted as in figure 5.7 below.

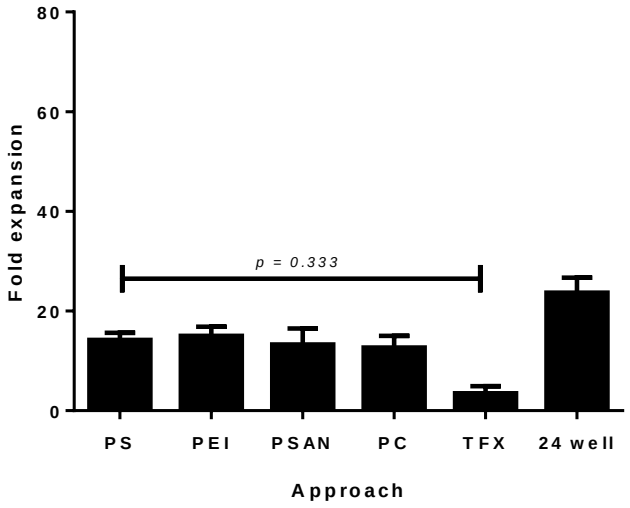


Figure 5.7: T-cell fold expansion on 5 materials with roughness R45 after 8 days of culture.
T-cells generated from fresh blood were cultured on five material cup inserts with rough surface (R45) for 8 days (see fig. 5.2). The 24-well plate culture was used for comparison. Fold expansion was calculated at the end of culture. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate and TFX = Tecoflex®. Statistical analysis was performed using Mann-Whitney test, n = 2 (this experiment repeated 2 times using 2 different healthy donors).

The material TFX again for the third time seems to have the worst fold expansion while the other four materials (PS, PEI, PSAN and PC) have similar fold expansions (average = 13.8). However, this time the growth in the four materials (PS, PEI, PSAN and PC) is less than the 24-well plate culture (which stands at approximately 23.7). This is surprising since this result is quite similar to the first experiment involving growth on R00 surfaces, figure 5.3 (page 20). Please also see the roughness testing experiments (page 39) where further investigations are done on the influence of roughness on T-cell growth. The figure 5.8 on the next page shows the appearance of T-cell cultures on roughness R45 on day 8 before harvest.

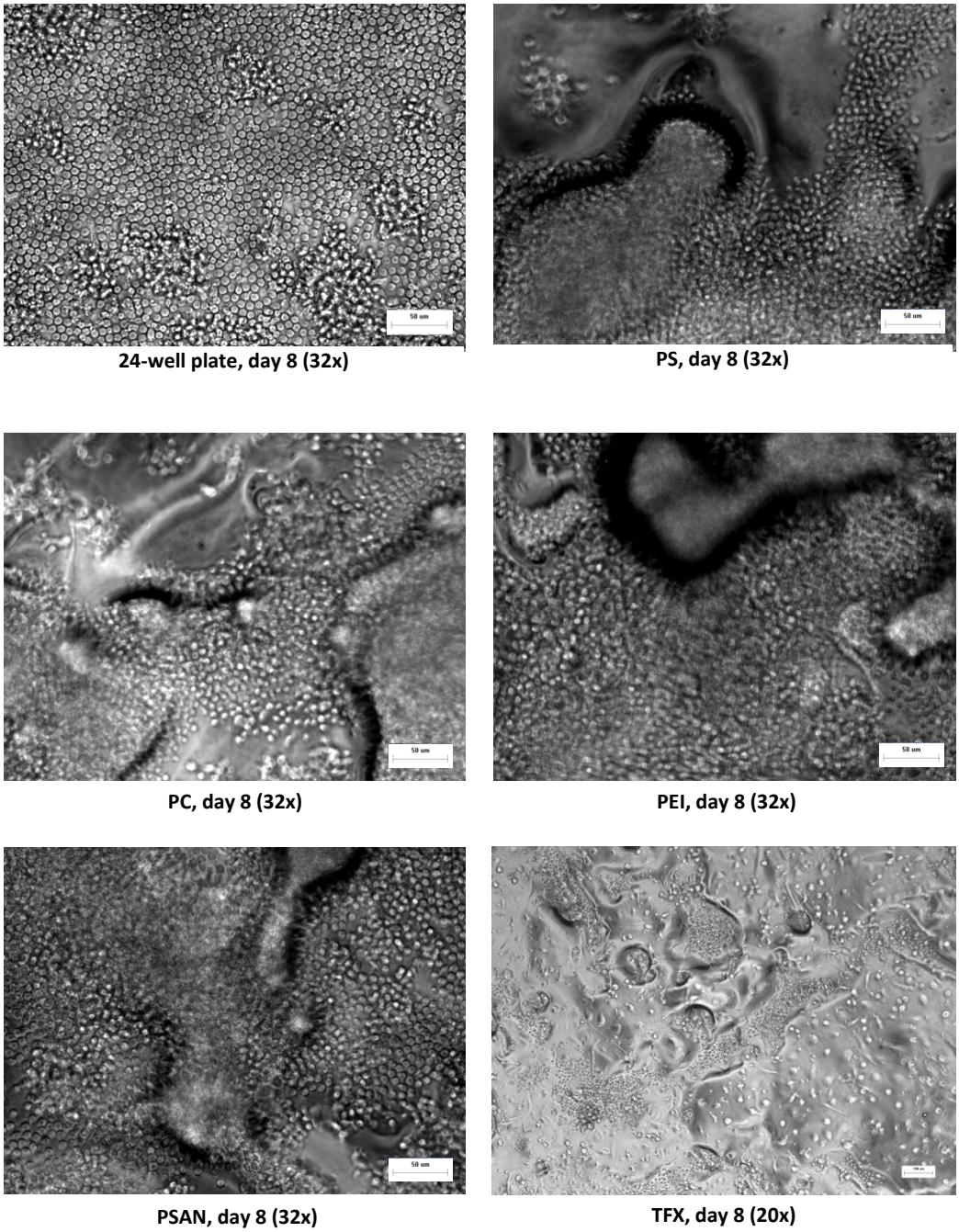


Figure 5.8: Appearance of T-cell cultures grown on R45 materials on day 8 before harvest.
T-cells generated from fresh blood were cultured on material cup inserts with rough surface (R45). The 24-well plate culture was used for comparison. At the end of culture pictures were taken with a light microscope. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. The scale is 50µm in the six pictures.

These images were taken with a light microscope (Axiovert 40CFL) from (Carl Zeiss Microscopy). While there was similar growth on the four materials (PS, PC, PSAN and PEI) the growth of T-cells on TFX (lowest right) was very poor. In order to identify what was happening with the T-cells grown on the TFX material we stained the cells for activation markers (TNFα and CD107a) as shown in the next experiment.

4. T-cell stain for activation markers after culture on 5 materials and 3 roughness grades:

The T-cells were generated using the EBV peptide stimulation protocol (please see: "*EBV peptide stimulation protocol*" in "*methods*", page 104) and cultured on five materials and three roughness for 18 days. After harvesting on the last day the cells were not stimulated but directly stained for activation markers (TNFα, CD107a and IFNγ) to observe any T-cell activation as a result of culture on the materials.

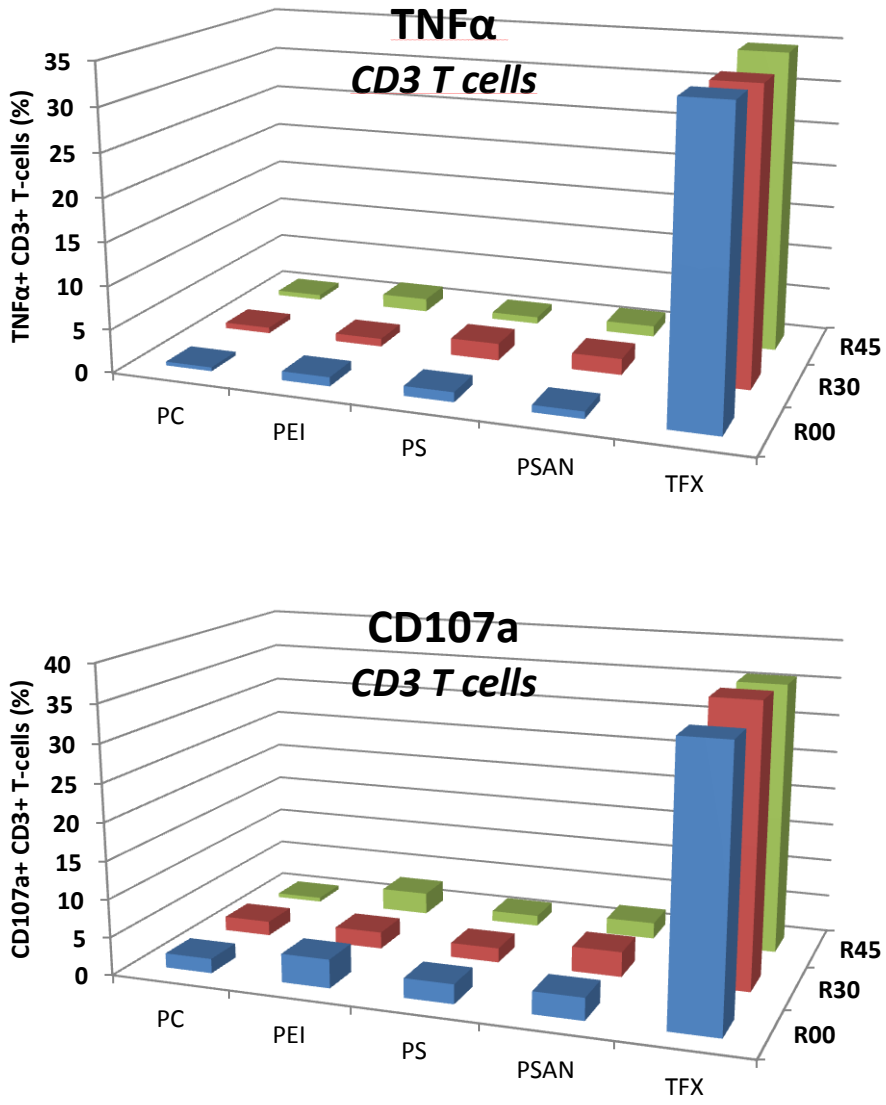


Figure 5.9: T-cells expressing TNFα and CD107a after extended culture on 5 materials with 3 roughness grades.
EBV specific T-cells generated from a buffy coat were cultured on material cup inserts. These were the five materials (PS, PSAN, PEI, PC and TFX) and the three grades of roughness (R00, R30 and R45). After culture for 18 days the cells were stained for surface markers CD3 and CD107a and cytokine marker TNFα. The percentage of live CD3+ cells that are expressing CD107a and TNFα was determined.

Among all the CD3+ T-cells measured there seems to be a very high proportion of cells expressing TNFα (33 to 34%) and CD107a (35.1 to 36.6%) when grown on TFX compared to other materials of same roughness grades (0.44 to 3.76%) in figure 5.9. None of these cells have been previously stimulated before the flowcytometry analysis was done. The induction of this activated state appears to be due to the material alone.

Interferon gamma (IFNγ) secretion was negligible (below 1% for all materials including TFX) and did not show a significant difference between any of the materials (graph not shown here).

5. T-cell function after culture for 18 days on 4 materials and 3 roughness grades:

Once it was clear that TFX could induce T-cell activation it was necessary to determine if overall T-cell functionality could be affected by the various materials tested. EBV specific T-cells were generated with the EBV peptide stimulation protocol. The eluted T-cells were cultured for 18 days on 4 materials and 3 roughnesses after which they were counted, re-stimulated with EBV peptide and stained for markers of functionality.

For these experiments TFX was not included in the investigation because they have been shown in the previous experiment to influence T-cell activation making it hard to investigate the actual T-cell functionality. Additionally TFX was also deemed not favourable for T-cell culture due to the low cell numbers seen in the last 4 experiments.

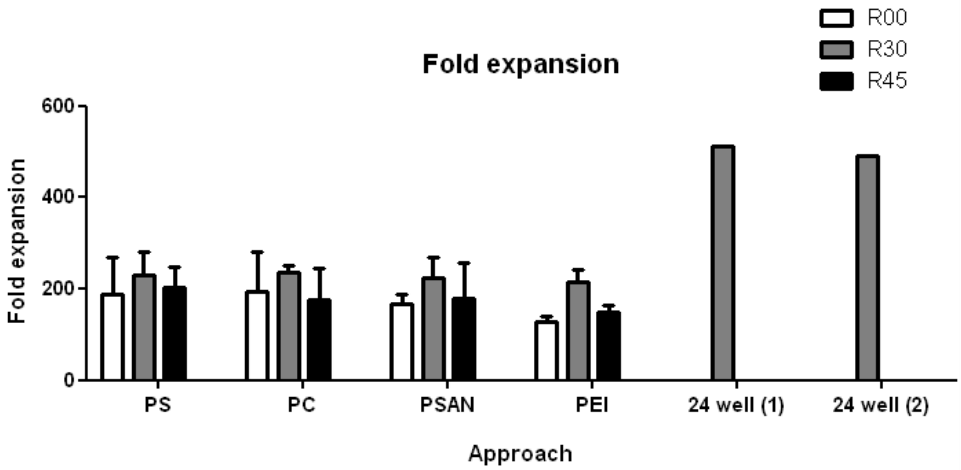


Figure 5.10: Fold expansion of T-cells after culture on 4 materials and 3 roughnesses for 18 days. *EBV specific T-cells generated from buffy coats were cultured on material cup inserts and 24-well plate for 18 days and then harvested and fold expansion determined. This experiment was repeated 2 times using buffy coats from 2 different healthy donors, each exposed to 4 materials of 3 roughness types respectively, including one 24 well plate for comparison. Materials tested were: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. Roughness grades used: R00 (white bars), R30 (grey bars) and R45 (black bars).*

It was found after cell number counting that there was similar fold expansion among all four materials within the same roughness grade (figure 5.10). However, there was a slightly higher fold expansion (216 to 234.6 fold) seen in roughness R30 compared to the other two (R00 and R45) roughness grades (127 to 203 fold). This was further investigated in roughness testing experiments (see page 39). Additionally another interesting finding is that the 24-well plate cultures showed the highest fold expansion (490, 512 fold) after 18 days of culture compared to the material cups.

Then in order to test functionality, the T-cells were next stimulated with EBV peptides and incubated for 6 hours with Brefeldine A and then stained for cytokine markers (TNFα and INFγ) and surface markers (CD4, CD8 and CD107a) as in the figures below and in following pages.

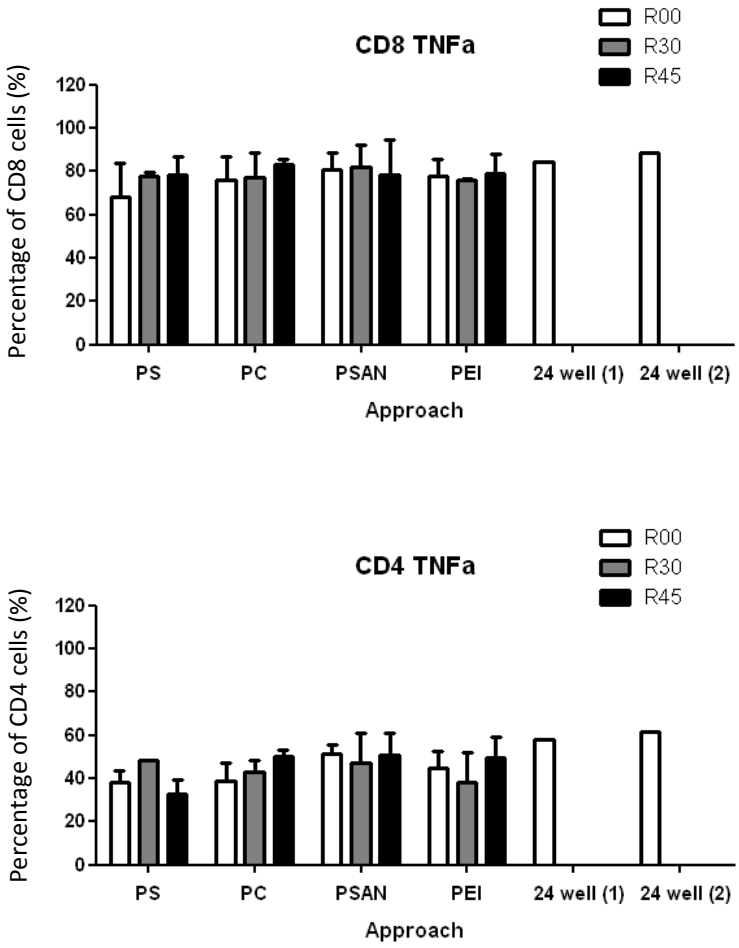


Figure 5.11: Percentage of T-cells producing TNFα after culture for 18 days culture.
EBV specific T-cells generated from buffy coats were grown on material cup inserts and 24-well plate and after culture they were stimulated with EBV peptide and stained for TNFα. This experiment was repeated 2 times using buffy coats from 2 different healthy donors, each exposed to 4 materials of 3 roughness types respectively, including one 24 well plate for comparison. Results were plotted as percentage of live CD4 or CD8 cells producing TNFα. Materials tested: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. Roughness grades used: R00 (white bars), R30 (grey bars) and R45 (black bars).

During flowcytometry analysis live CD4 and CD8 populations of T-cells were gated and the cytokine productions from these cells were determined. There was similar production of TNFα among all four materials (PS, PC, PSAN and PEI) and 24-well plate cultures in both CD4 and CD8 T-cells as in figure 5.11. Roughly 40 to 50% of the CD4 cells and a higher 70 to 80% of CD8 cells were expressing TNFα.

This was also the same case with IFNγ production too as seen in the figure 5.12, where the levels were the same irrespective of the roughness, material or size of culture chamber. Approximately 30 to 40% of the CD4 cells and a higher 70 to 80% of CD8 cells secreted IFNγ after re-stimulation.

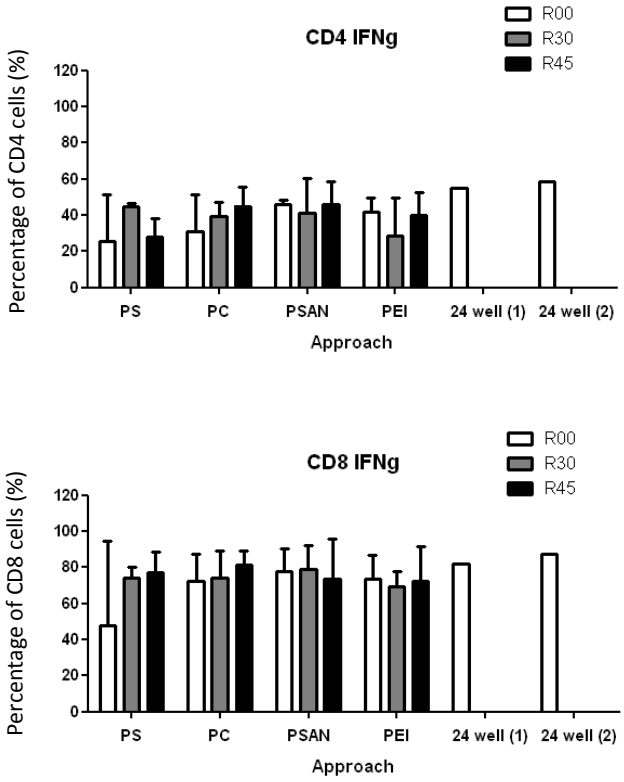


Figure 5.12: Percentage of T-cells producing IFNγ after culture for 18 days culture.
EBV specific T-cells generated from buffy coats were grown on material cup inserts and 24-well plate and after culture they were stimulated with EBV peptide and stained for IFNγ. This experiment was repeated 2 times using buffy coats from 2 different healthy donors, each exposed to 4 materials of 3 roughness types respectively, including one 24 well plate for comparison. Results were plotted as percentage of live CD4 or CD8 cells producing IFNγ. Materials tested: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. Roughness grades used: R00 (white bars), R30 (grey bars) and R45 (black bars).

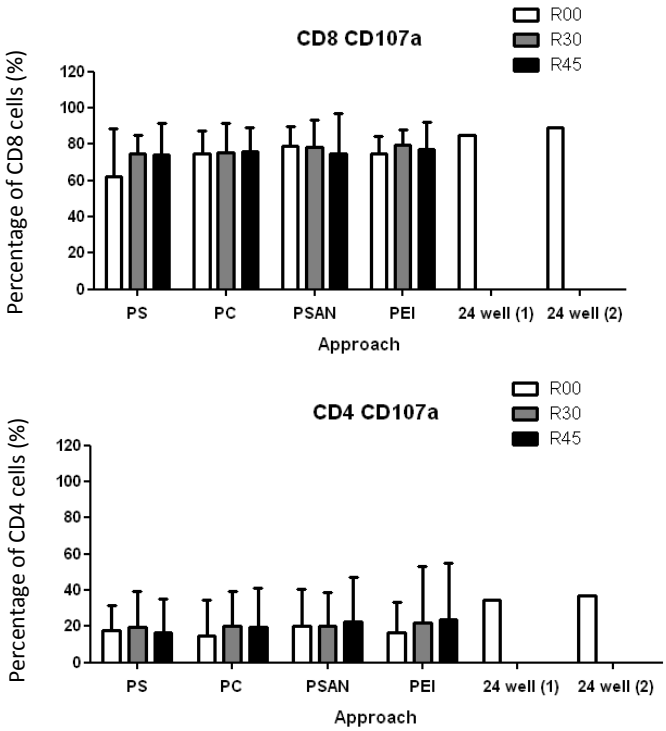


Figure 5.13: Percentage of T-cells expressing CD107a after exposure to EBV peptides (culture for 18 days).

EBV specific T-cells generated from buffy coats were grown on material cup inserts and 24-well plate and after culture they were stimulated with EBV peptide and stained for IFNγ. This experiment was repeated 2 times using buffy coats from 2 different healthy donors, each exposed to 4 materials of 3 roughness types respectively, including one 24 well plate for comparison. Results were plotted as percentage of live CD4 or CD8 cells expressing CD107a. Materials tested: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. Roughness grades used: R00 (white bars), R30 (grey bars) and R45 (black bars).

Then staining for the marker for de-granulation on both CD4 and CD8 T-cells after stimulation with EBV peptides demonstrated (figure 5.13) that CD107a was also uniformly expressed on all the cells irrespective of the material, roughness or geometry. There was however a difference in the percentage of CD4 and CD8 cells expressing CD107a, where 20 to 25% of CD4 cells were expressing CD107a compared to a higher 70 to 80% in CD8 cells.

The proportion of live CD4 and CD8 cells among the T-cells cultured on all approaches were also investigated as shown in the figures below:

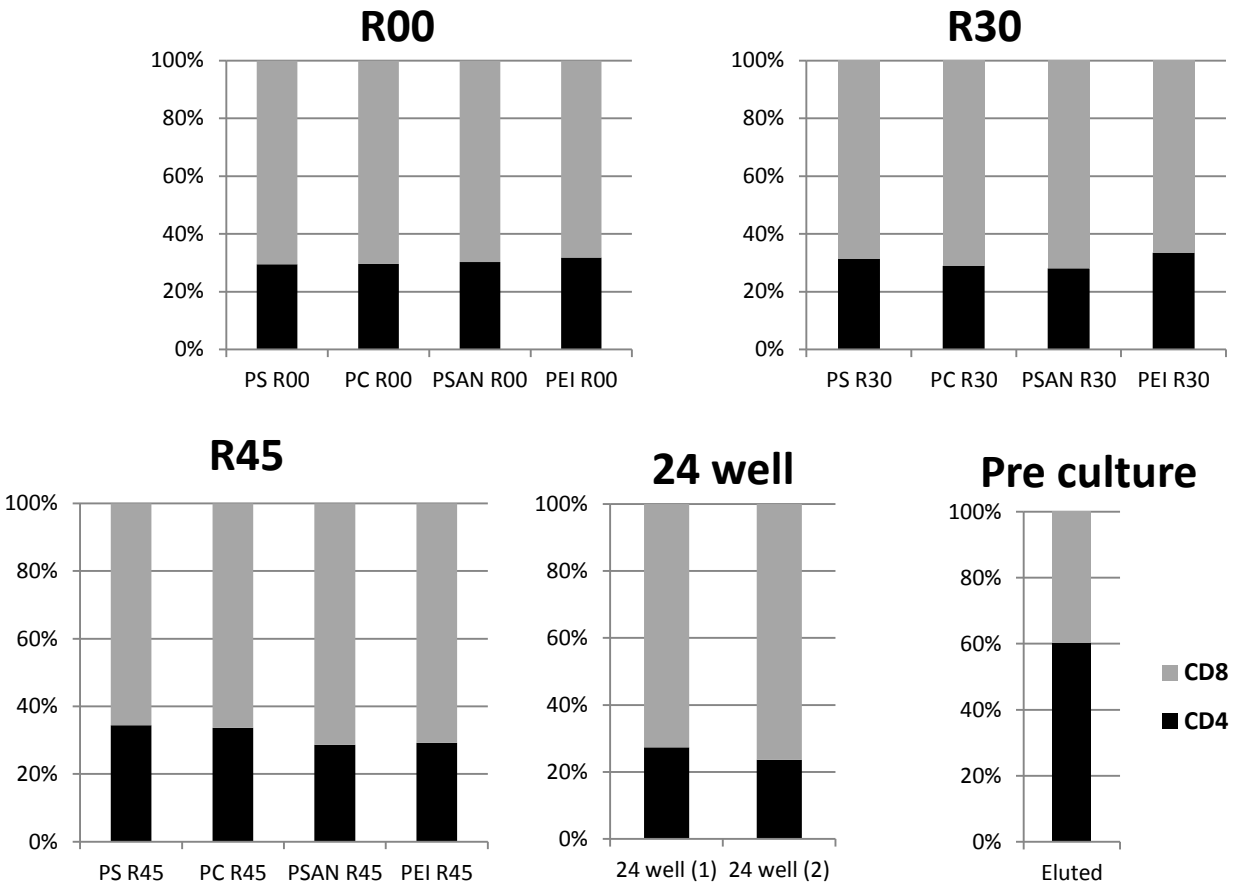


Figure 5.14: Proportion of CD4 and CD8 cells in the T-cells grown for 18 days on 4 materials and 3 roughnesses

EBV specific T-cells generated from a buffy coat were grown on material cup inserts and 24-well plate and after culture were stained for CD4 and CD8 cells. Results were plotted as proportion of live CD4 (black) and CD8 (grey) cells measured. Materials tested: PS = Polystyrene, PSAN = Poly (styrene-co-acrylonitrile), PEI = Poly (ether imide), PC = Polycarbonate, TFX = Tecoflex®. Roughness grades used: R00, R30 and R45.

The pre-culture proportions were determined by staining a sample of cells on day 0 before plating the cells in the various material inserts. During flowcytometry analysis, only live CD4 and CD8 cells were counted from pre-culture and post-culture stains, however the dead cell proportion was small and negligible compared to live cell proportion. It appears that CD4 and CD8 cell proportions are very similar among all the approaches tried in this experiment. This similarity in CD4 and CD8 proportion is irrespective of the material or roughness or size of culture chamber (since 24-well plate cultures are larger than the material inserts). Pre-culture levels indicate a slightly higher proportion of CD4 cells (60%) compared to after culture stains.

Section 2:

T-cell culture chamber interactions

T-cell culture chamber interactions

The culture chamber was next investigated which possibly could have an influence on T-cell growth. These experiments were carried out with the use of commercially available culture plates (please see: "*Geometry testing (plates + approaches)*" in "*methods*", page 111) and a set of "approaches" were formulated to investigate if a sudden or gradual change in geometry (shape and dimensions) of the culture chamber had an influence on T-cell expansion during the initial stages of culture. The figure 5.15 below is a reminder of the approaches used in experiments.

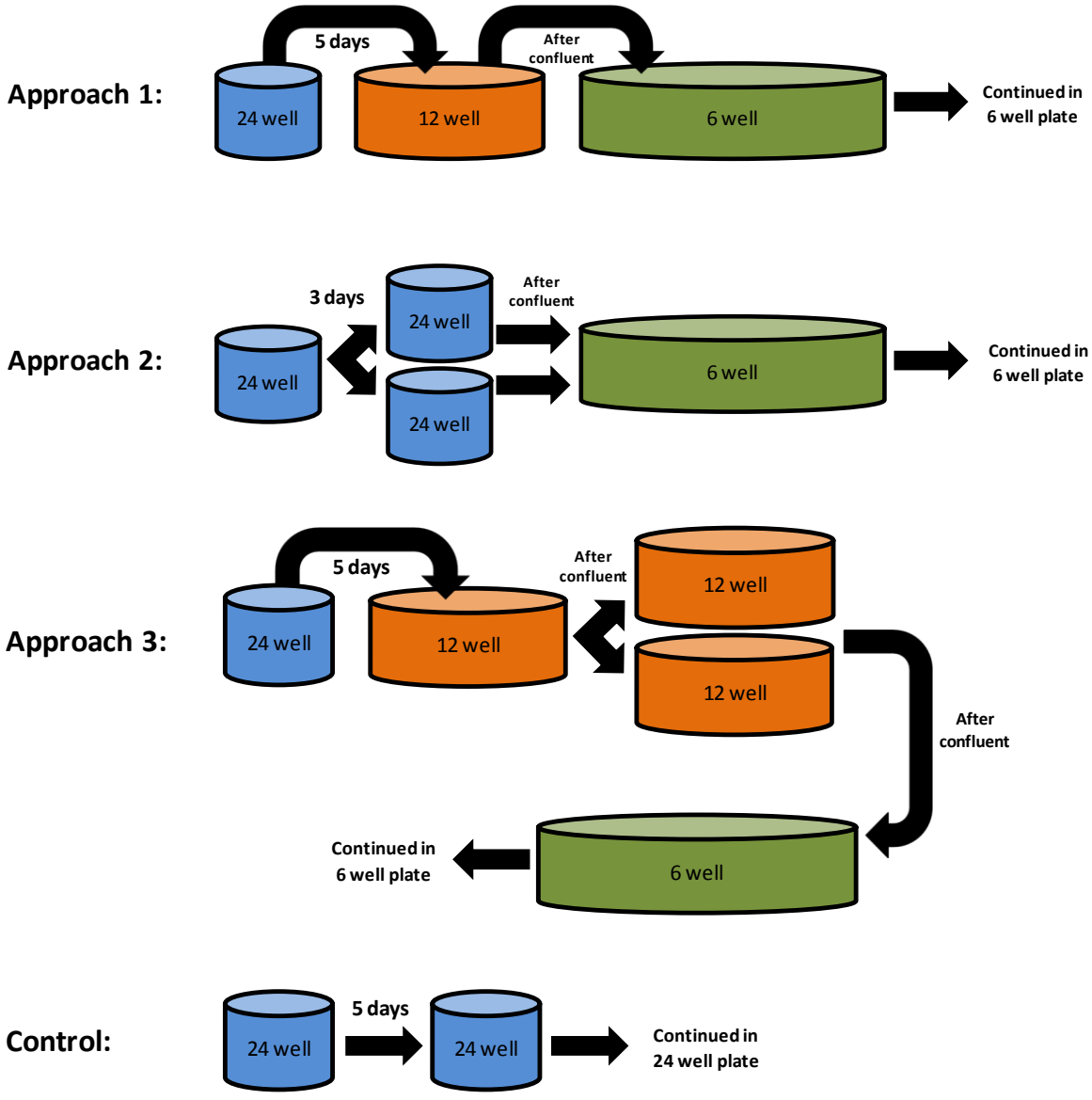


Figure 5.15: Approaches used in geometry testing experiments.
Geometry testing approaches were formulated using 24-. 12- and 6-well plates to represent "sudden" or "gradual" changes in geometry during T-cell culture.

1. Geometry testing experiments:

EBV specific T-cells were cultured for 18 days using the EBV peptide stimulation protocol in the four approaches (see previous page) and at end of culture the cells were counted and analysed for function and maturation states. The growth of T-cells after culture is as in figure 5.16 below:

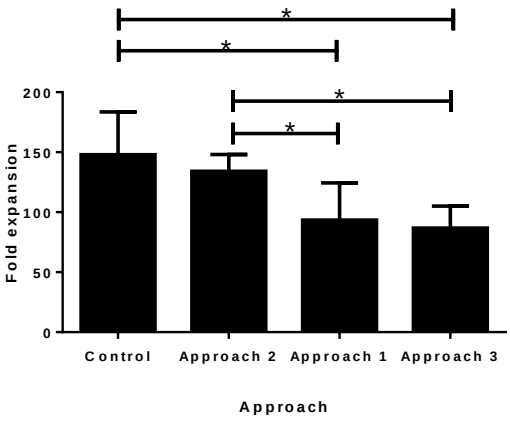


Figure 5.16: T-cell fold expansion in 3 approaches and 24-well plate control after 18 days of culture. *EBV specific T-cells generated from buffy coats were cultured in four approaches and harvested after 18 days and fold expansion determined. Statistical analysis was performed using Wilcoxon test. n = 6 (this experiment repeated 6 times using buffy coats from 6 different healthy donors); *p<0.01*

Out of the four approaches, significantly higher expansion was seen in T-cells growing in 24-well plate control and approach 2 (147 and 134 respectively). The approaches 1 and 3 (93 and 86 respectively) were significantly lower than both the 24-well plate control and approach 2. On further investigation the surface area used up by all the approaches was also plotted for day 18 (last day of culture) and the figure 5.17 illustrates surface area utilization in each approach. Culture surface area was calculated by the number of wells occupied per approach on the last day of culture.

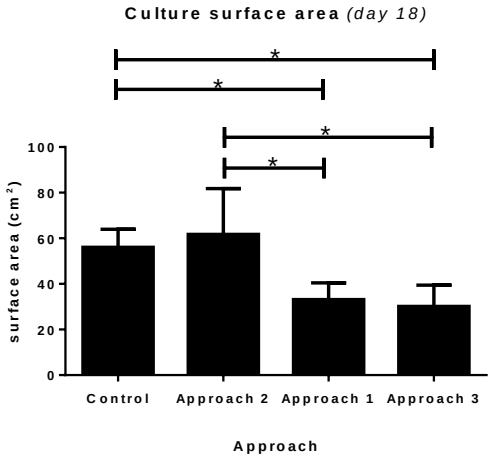


Figure 5.17: Culture surface area occupied by T-cells on day 18 (last day) of culture.
EBV specific T-cells generated from buffy coats were cultured in four approaches and harvested after 18 days and surface area occupied was determined by counting number of confluent wells per approach on last day of culture. Statistical analysis was performed using Wilcoxon test.
*n = 6 (this experiment repeated 6 times using buffy coats from 6 different healthy donors); *p<0.01*

It appears that a significantly higher amount of surface area is occupied by the T-cells grown in 24-well plate control and approach 2 (56 and 61 cm^2 respectively) compared to the other two approaches 1 and 3 (33 and 30 cm^2 respectively). The slightly higher value in approach 2 is because of an outlier which otherwise has similar growth to the 24-well control.

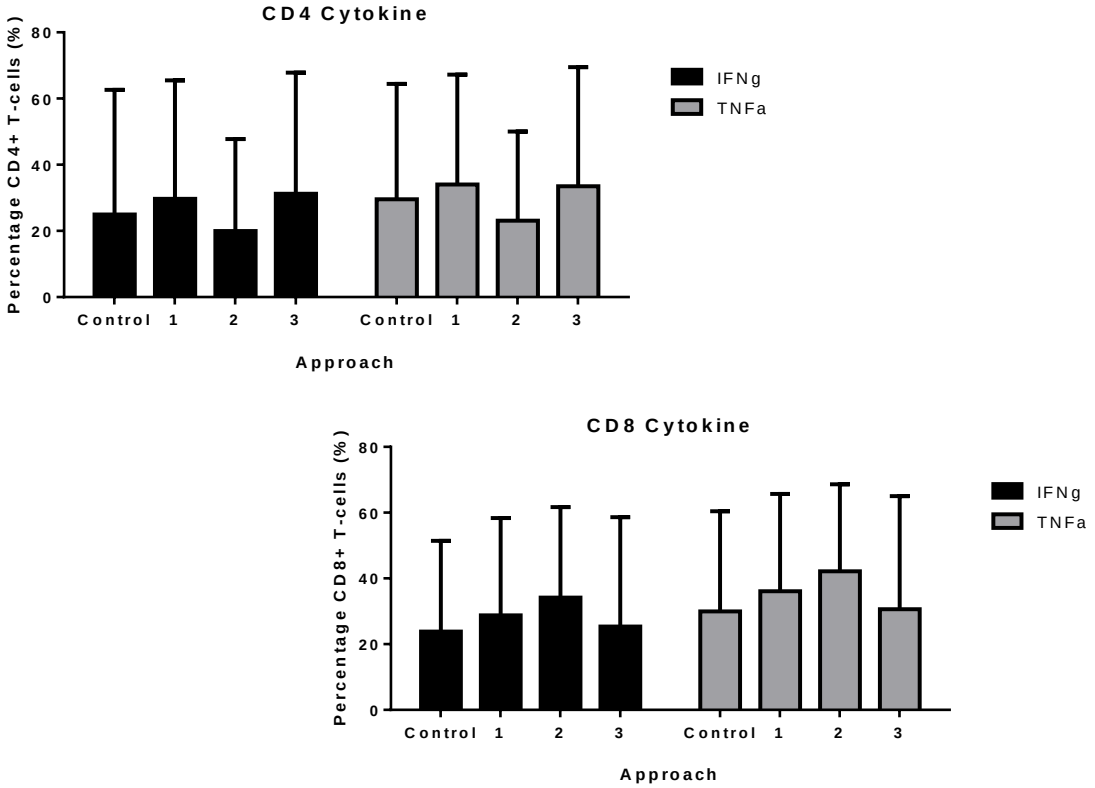


Figure 5.18: Cytokine production in T-cells grown in all approaches and control for 18 days.
EBV specific T-cells generated from buffy coats were grown in the four approaches and harvested on day 18 and re-stimulated with EBV peptide and stained for cytokine markers INFγ (black bars) and TNFα (grey bars). The cells were also stained for surface markers CD4 and CD8. n = 4 (this experiment repeated 4 times using buffy coats from 4 different healthy donors).

Next the functionality of the T-cells was investigated by looking at the cytokine secretion (IFNγ and TNFα) and surface expression of CD107a. The T-cells from the four approaches were stimulated with EBV peptide and incubated for 6 hours with Brefeldine-A. They were then stained for surface markers CD4 and CD8 and cytokine markers IFNγ and TNFα.

There seems to be comparable cytokine production among the four approaches (figure 5.18). The IFNγ production in CD4 cells was between 19 to 31% while CD8 cells were from 23 to 34%. The TNFα production was between 23 to 34% in CD4 cells while CD8 cells reached between 29 to 42%. The differences between approaches were not found to be statistically significant.

There was also no difference seen in CD107a expression (marker for degranulation) after stimulation with EBV peptides (figure 5.19). Around 39 to 47% of CD8 cells expressed CD107a while around 11 to 17% of CD4 cells expressed CD107a.

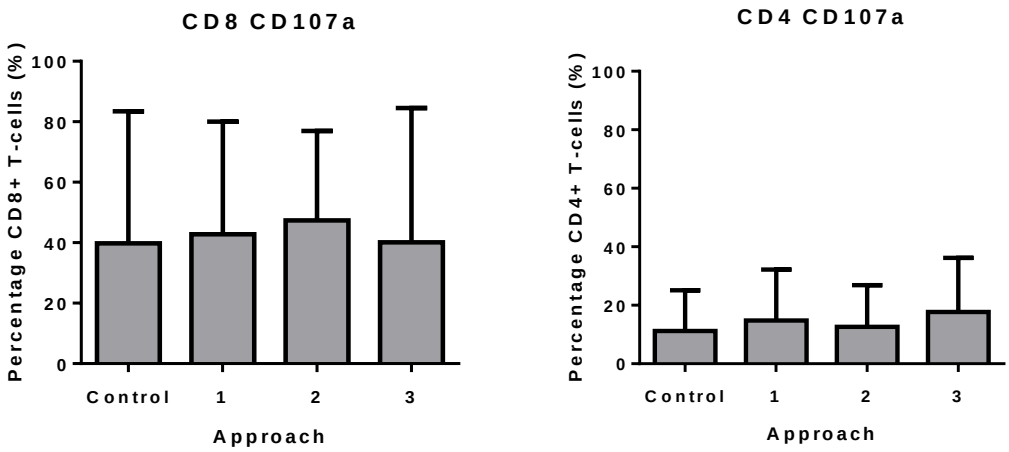


Figure 5.19: CD107a expression in T-cells grown in all approaches and control for 18 days.
EBV specific T-cells generated from buffy coats were grown in the four approaches were harvested on day 18 and re-stimulated with EBV peptide and stained for surface markers CD4, CD8 and CD107a. n = 4 (this experiment repeated 4 times using buffy coats from 4 different healthy donors)

Since the geometry size can influence cytokine availability and diffusion; this could also influence the maturation of T-cells. Hence we next investigated the maturation of the T-cells grown on all the approaches (figure 5.20).

Maturation was determined by staining for surface markers CD45RA and CCR7. T-cell maturation was classified as: Effector memory (CD45RA- and CCR7-), Central memory (CD45RA- and CCR7+) and Effectors (CD45RA+ and CCR7+). It appears that the maturation of

CD4 and CD8 cells was the same across all approaches and control as in figure 5.20 with a major population of Effector memory cells seen in both CD4 and CD8 cells (86 to 95%). There were also a slightly higher proportion of effector cells in CD8 cells (12 to 13.5%) compared to CD4 cells (2.9 to 3%) among the T-cells measured. There was a very low proportion of central memory in CD4 cells (1 to 4.2%) and almost no central memory in CD8 cells.

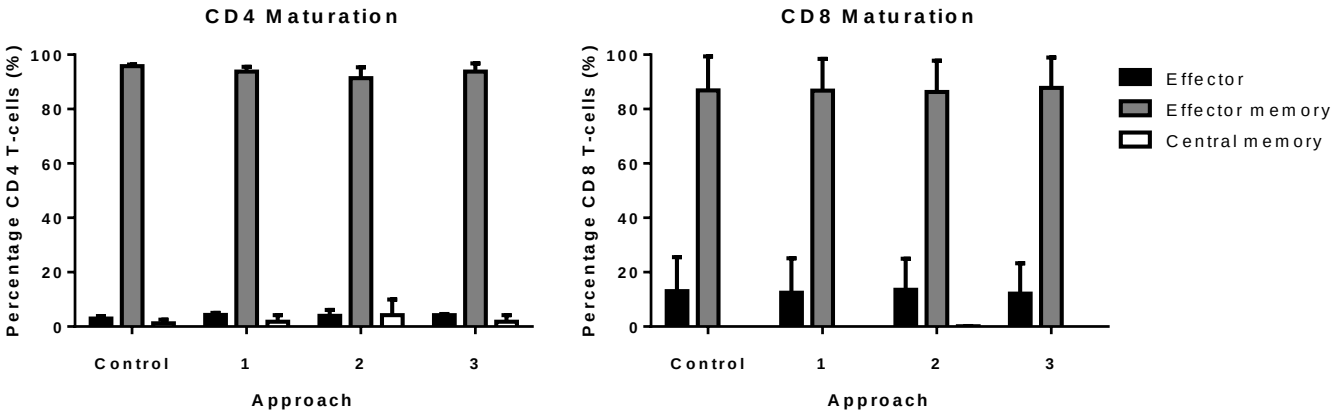


Figure 5.20: Maturation states of T-cells grown on all approaches and control for 18 days.
EBV specific T-cells generated from buffy coats were cultured in the four approaches were stained for markers of maturation (CD45RA and CCR7) after harvest on day 18. In the figure, black bars are effector memory (CD45RA- and CCR7-), white bars are Central memory (CD45RA- and CCR7+) and grey bars are Effectors (CD45RA+ and CCR7+). n = 2 (this experiment repeated 2 times using buffy coats from 2 different healthy donors).

2. Roughness testing experiments:

After geometry testing experiments the next component of the culture chamber, "culture surface roughness" was investigated in the following set of roughness testing experiments.

The aim of roughness testing experiments was to further investigate roughness influence on T-cell expansion. As it was seen previously in the material testing experiments, rough surfaces seem to have an influence on T-cell growth. The following experiments were also carried out using material cup inserts which were physically modified to have rough surfaces (please see "Material cups and roughness" in "methods", page 114). The experimental set-up was as shown in figure 5.21.

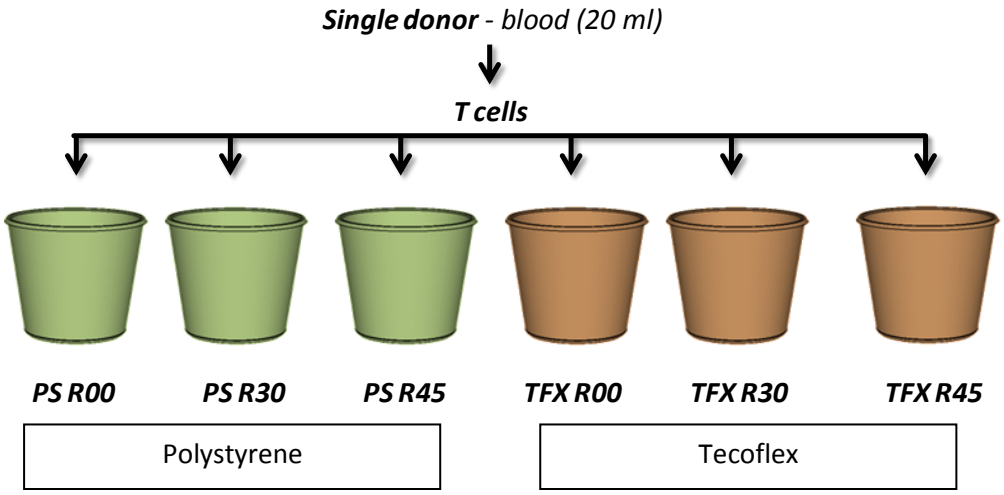


Figure 5.21: Roughness testing experiments using Pan T-cell stimulation protocol.
The three grades of roughness (R00, R30 and R45) were used and two materials (PS and TFX). Pan T-cell stimulation protocol was used to isolate T-cells and culture was carried out for 8 days.

T-cells were isolated from fresh blood using the pan T-cell stimulation protocol. These cells were cultured for 8 days in all approaches in figure 5.21 and at the end cell numbers determined after harvesting (figure 5.22, next page).

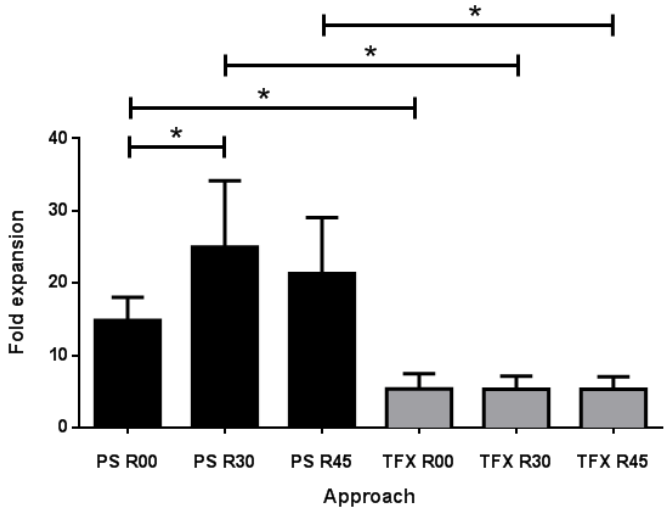


Figure 5.22: Fold expansion of T-cells after culture in 3 grades of roughness for 8 days using the pan T-cell stimulation protocol with Materials PS and TFX.
*The three grades of roughness (R00, R30 and R45) were used and two materials (PS and TFX). Culture was carried out for 8 days using T-cells generated from fresh blood (see fig. 5.21) using the pan T-cell stimulation protocol. The fold expansion was determined on day 8 of culture after harvest. Statistical analysis was performed using Wilcoxon test. n = 6 (this experiment repeated 6 times using fresh blood from 6 different healthy donors); *p<0.01*

As seen in previous experiments (Material testing pages 20, 22, 24), there is a significantly higher fold expansion in T-cells grown on PS compared to TFX of corresponding roughness grades. Among the PS approaches the T-cells seem to demonstrate better growth on rough surfaces compared to smooth. Out of the three surfaces in polystyrene (PS R00, PS R30 and PS R45) there is significantly higher expansion of T-cells on R30 (24.9 fold) compared to smooth surfaces R00 (14.8 fold) and R45 (21.3 fold) making R30 to have the highest overall fold expansion. In contrast, the T-cells on TFX show no such difference in growth irrespective of the roughness (5.35 to 5.32 fold).

This experiment was repeated again but this time T-cells were generated using the EBV peptide stimulation protocol (figure 5.23). The T-cell culture was carried out for 18 days on the same set of approaches and at the end the cells were harvested, counted and analysed for functionality.

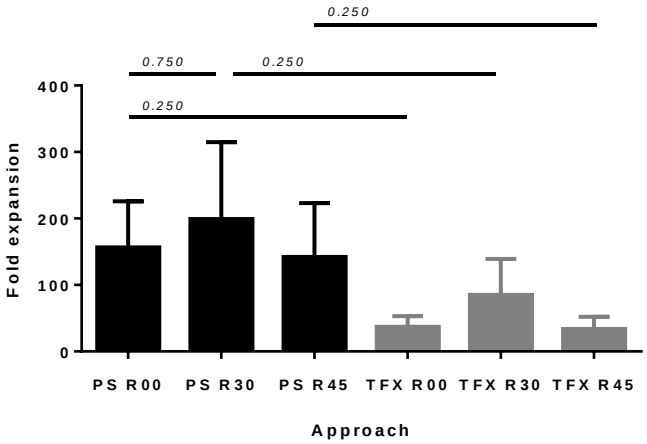


Figure 5.23: Fold expansion of T-cells after culture in 3 grades of roughness for 18 days using the EBV peptide stimulation protocol with Materials PS and TFX.
The three grades of roughness (R00, R30 and R45) were used and two materials (PS and TFX). Culture was carried out for 18 days with T-cells generated from buffy coats using the EBV peptide stimulation protocol (see fig. 5.21). The fold expansion was determined on day 18 of culture after harvest. n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors). Statistical analysis done via Mann-Whitney U test.

On day 18 after harvest the T-cells showed a higher fold expansion in PS (142 to 199 fold) compared to TFX (33 to 85 fold) and the roughness R30 still shows the highest fold expansion at 199 on polystyrene (figure 5.23). However, this time R30 is also the highest in TFX reaching 85 fold compared to TFX R00 and TFX R45 (37 and 33 fold respectively). Additionally the growth of T-cells on PS R00 has been able to catch-up to the growth in PS R45. This profile (where R30 is highest and R00 and R45 are almost equal) is seen in both materials PS and TFX after 18 days of culture. This kind of growth was not seen in the previous experiment which involved 8 days of culture (figure 5.22).

Next in order to analyse functionality, the EBV specific T-cells grown on the rough surfaces in figure5.23 (previous page) were r e-stimulated with EBV peptides and incubated for 6 hours with Brefeldine-A and then stained for cytokine markers (IFNγ and TNFα) and surface marker for degranulation CD107a.

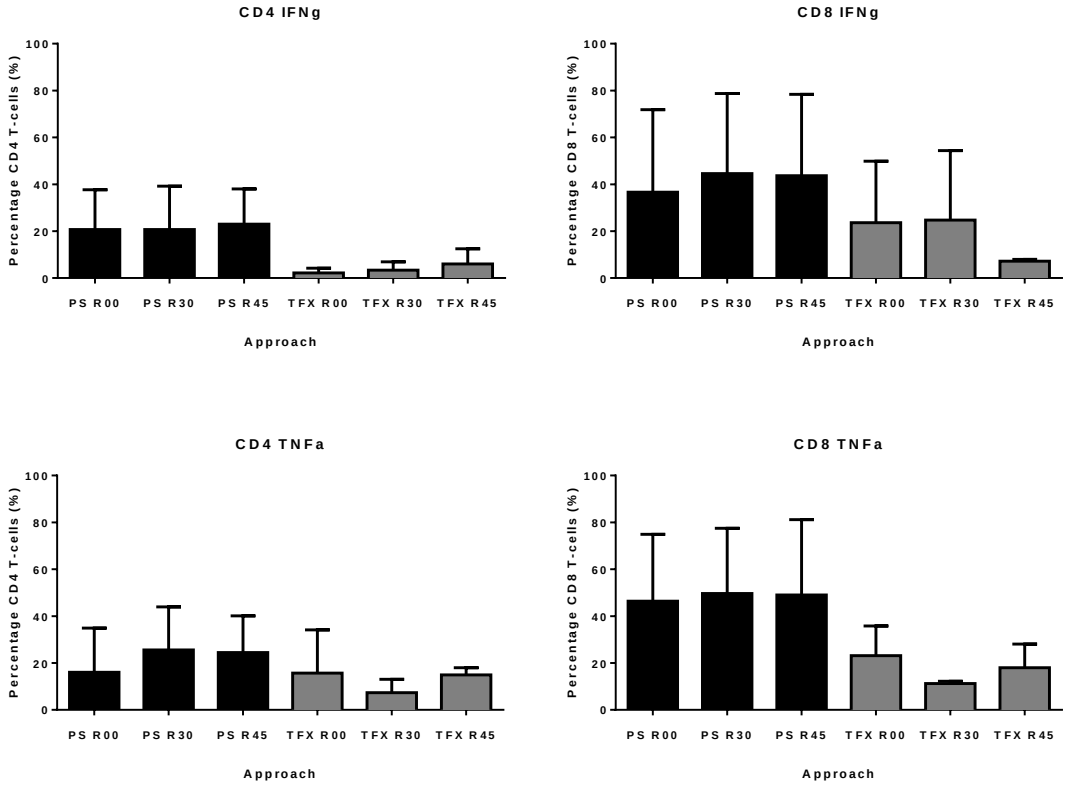


Figure 5.24: IFNγ and TNFα production in T-cells cultured for 18 days on three grades of roughness. *EBV specific T-cells generated from buffy coats were grown on the two materials (see fig. 5.21), PS (black bars) and TFX (grey bars) and were harvested on day 18 and re-stimulated with EBV peptide and stained for cytokine markers INFγ and TNFα. The cells were also stained for surface markers CD4 and CD8. Three grades of roughness were tested (R00, R30 and R45). n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors).*

During flowcytometry analysis live CD4 and CD8 cells were gated and cytokine and CD107a expression was analysed. The amount of cytokine secreted by stimulated cells does not seem to differ if the cells are grown on smooth or rough surfaces (figure 5.24); however, the cells grown on PS produced more cytokine than those grown on TFX.

The IFNγ production in CD4 cells grown on polystyrene reached around 20 to 23% while those on TFX reached only 2 to 6%. In the case of CD8 cells IFNγ secretion reached 36 to 44% on polystyrene while only 7 to 24% of CD8 cells responded after culture on TFX. When investigating TNFα production in CD4 cells, roughly 16 to 25% cells responded when grown on polystyrene while 7 to 15% responded in TFX cultures. The CD8 cells TNFα secretion was

around 46 to 49% when grown on polystyrene while only 11 to 23% CD8 cells responded when grown on TFX.

Next analysing CD107a expression around 11 to 15% of CD4 cells grown on polystyrene responded while only 1 to 4.9% CD4 cells responded when grown on TFX (figure 5.25). The CD8 cells showed a big ger difference where 55 to 65% of cells responded when grown on polystyrene compared to 7 to 14% on TFX.

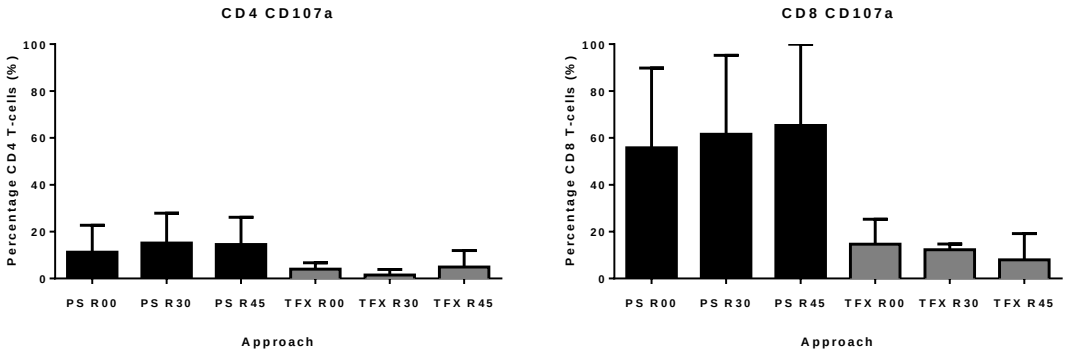


Figure 5.25: CD107a expression on T-cells cultured for 18 days on three grades of roughness.
EBV specific T-cells generated from buffy coats were grown on the two materials (see fig. 5.21), PS (black bars) and TFX (grey bars) and were harvested on day 18 and re-stimulated with EBV peptide and stained for surface markers CD4, CD8 and CD107a. n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors).

In a separate investigation when analysing the cytokine secretion in cells grown on rough and smooth surfaces for multi-producers (cells that are producing all three: IFNγ, TNFα and CD107a at the same time) there are significantly higher proportions of multi-producers among T-cells grown on rough surfaces compared to smooth surfaces in the material PS (figure 5.26). This was not the case for the material TFX where most of the cells were found to be single producers (result not shown).

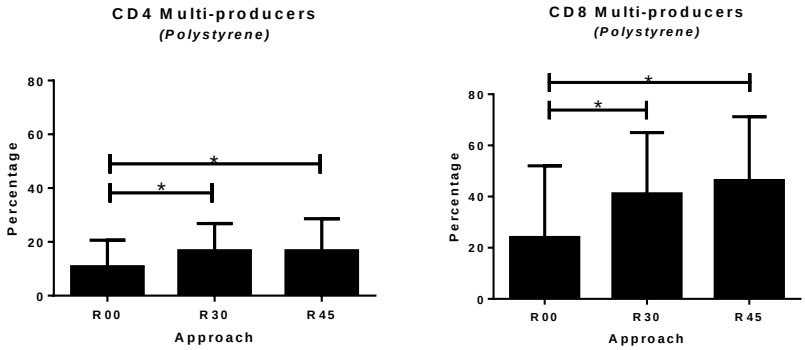


Figure 5.26: Multi-producers in T-cells grown on polystyrene (R00, R30 and R45).
*EBV specific T-cells generated from buffy coats were grown on polystyrene with three grades of roughness (R00, R30 and R45) were re-stimulated with EBV peptides after culture for 18 days and analysed for multi-producers. Multi-producers are cells capable of secreting IFNγ, TNFα and expressing CD107a at the same time. Statistical analysis was performed using Wilcoxon test. n = 6 (this experiment repeated 6 times using buffy coats from 6 different healthy donors); *p<0.01*

There was roughly the same amount of CD4 multi-producers on R30 and R45 (16%) while R00 had 10% multi-producers (figure 5.26). Again There was a higher amount of multi-producers among CD8 cells grown on R30 and R45 (41% and 46% respectively) while around 24% of the CD8 cells were multi-producers on R00.

3. Electrospun material testing experiments:

After the roughness testing experiments further experiments were carried out on a different type of surface called “electrospun material”. These are microfibers that are spun from the same materials used to manufacture the material cup inserts. The bottom of the cup is modified with electrospun fibres. Two types of alignments were used for testing: parallel and random aligned as in figure 5.27 below.

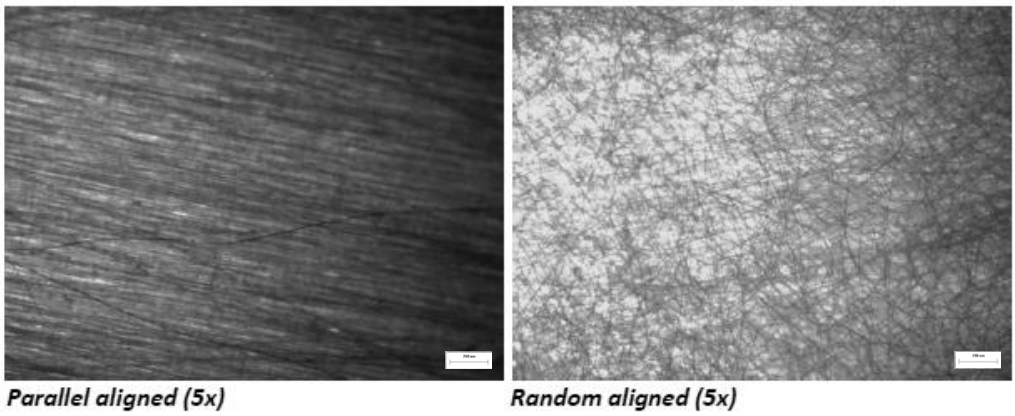


Figure 5.27: Parallel and random aligned electrospun fibres used in experiments.
These images were taken with a light microscope (Axiovert 40CFL) from (Carl Zeiss Microscopy). The scale is 200µm in the two pictures.

The pattern of surface cannot be fixed in the case of electrospun fibres due to the random nature of the electro-spinning process. However, the number of layers and density of the fibres can be modulated and fixed for all approaches. The experimental set-up we used for testing was as in figure 5.28 (next page).

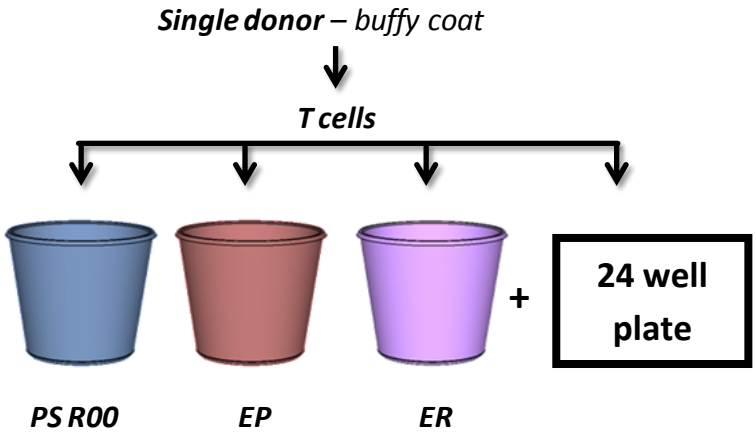


Figure 5.28: Electrospun material testing experiment set-up.
Parallel aligned (EP) and random aligned (ER) were compared to smooth material (PS R00) and 24-well plate culture. All materials are made of polystyrene material.

In these experiments the 24-well plate cannot be used as a control because of the differences in the size of geometry and oxygen plasma surface treatment to the material cup inserts. The 24-well plates were used only as a comparison to the other approaches.

Antigen specific T-cells were generated via the EBV peptide stimulation protocol and then the cells were cultured for 18 days on the four approaches. The following is the growth seen after harvest on day 18 in figure 5.29 below.

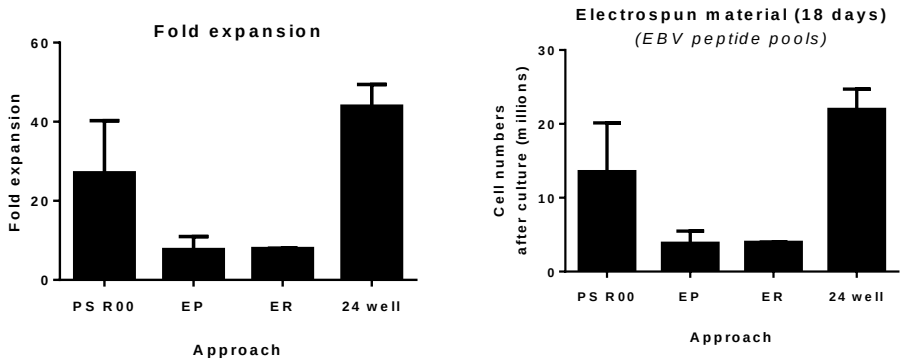


Figure 5.29: Fold expansion and final cell numbers of T-cells grown on electrospun materials
EBV specific T-cells generated from buffy coats were cultured on electrospun materials (EP = parallel and ER = Random aligned), smooth material (PS R00) and 24-well plate cultures for 18 days (see fig. 5.28). After harvest, cells were counted and fold expansion calculated. n = 2 (this experiment repeated 2 times using buffy coats from 2 different healthy donors)

The growth of T-cells on electrospun materials (parallel (EP) and random (ER) alignment) was very low compared to the smooth material insert (PS R00) and 24-well plate culture. The cells growing on EP reached a fold expansion of 7.7 which was similar to the cells growing on ER (7.9 fold). On the other hand the cells growing on PS R00 reached a fold expansion of 27

while the 24-well plate cultures had the highest fold expansion of 43.9. There was also no major difference in the proportions of CD4 and CD8 cells before and after 18 days of culture on electrospun materials and other approaches (figure 5.30).

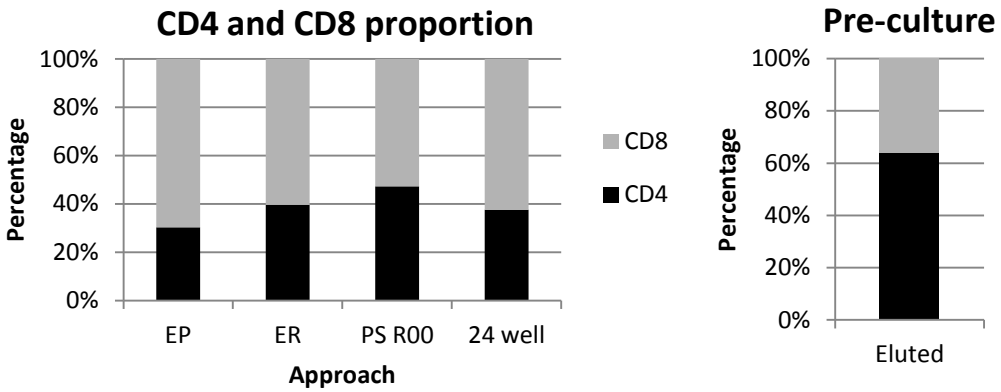
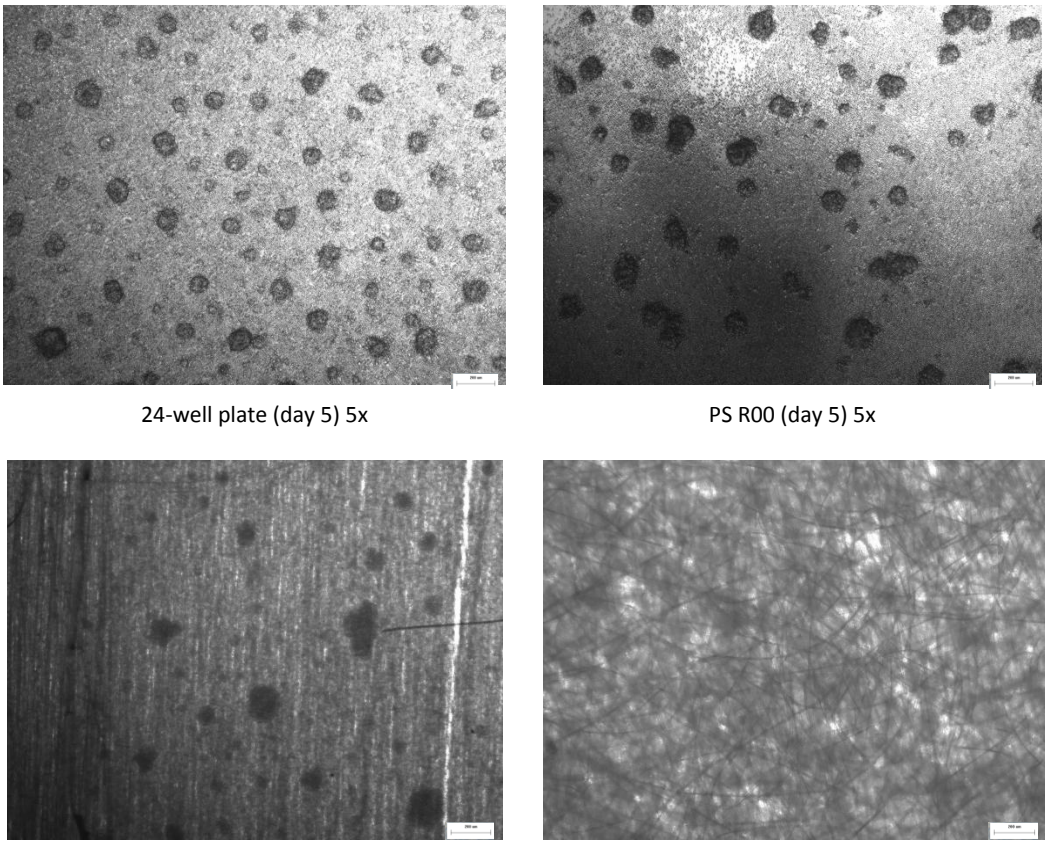


Figure 5.30: CD4 / CD8 proportions before and after culture on electrospun materials for 18 days. *EBV specific T-cells generated with buffy coats were cultured in the four approaches (see fig. 5.28) and cells were stained for CD4 and CD8 markers before and after 18 days of culture.*

The figure 5.31 below shows the appearance of T-cell clumps on all four approaches on day 5 of culture.



24-well plate (day 5) 5x

PS R00 (day 5) 5x

Electrospun Parallel (day 5) 5x

Electrospun Random (day 5) 10x

Figure 5.31: T-cell clumps appearance on electrospun materials, smooth materials and 24-well plate culture at day 5 of culture. *Pictures were taken with a light microscope (Axiovert 40CFL) from Carl Zeiss Microscopy. The scale is 200µm in the four pictures.*

It is clear that clumps have begun to form on the parallel aligned electrospun material while there are no visible clumps formed on the random aligned material. After day 5 the electrospun materials became dark and it was not possible to observe cell clumps under a light microscope. Also there seems to be a higher number of clumps forming on the smooth material (PS R00) and 24-well plate culture compared to electrospun materials on day 5.

Section 3:

T-cell and observer interaction

T-cell expansion technique

The expansion procedure used during T-cell culture is the next factor that can have an influence on the growth of cells. This depends on the observation of culture conditions (confluence and medium colour) and requires the observer to make a judgement of how to proceed with the next steps of culture.

The aim of these experiments was to develop a standard method of T-cell manipulation during culture (such as splitting cultures or changing medium). The final output should be predictable and controllable instead of being influenced by donor and other variables such as human judgement.

1. Cell number experiments:

Normally T-cell expansion is carried out for 18 days using the EBV peptide stimulation protocol. This results in a final cell number that varies depending on the donor after 18 days of culture as in figure 5.32 below.

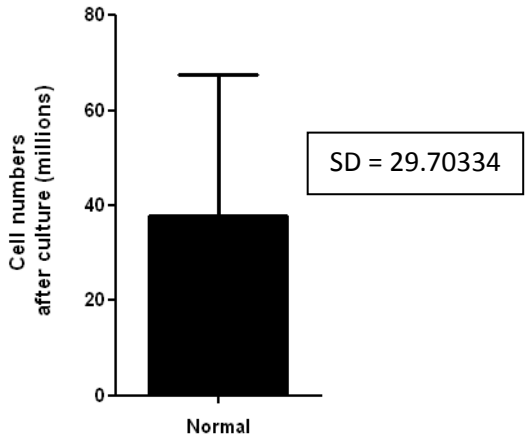


Figure 5.32: Average number of T-cells generated after 18 days of culture using the EBV peptide stimulation protocol.
EBV specific T-cells generated from fresh blood were cultured in 24-well plates for 18 days and then harvested and final cell number determined. Next standard deviation between donors was also calculated. n = 7 (this experiment repeated 7 times using fresh blood from 7 different healthy donors).

The standard deviation is approximately 29.7 in this group of donors with an average final cell number of 37 million (figure 5.32). A fixed splitting schedule was proposed (see “fixed splitting schedule” in “methods”, page 117) where T-cells were expanded at fixed time points. There was no direct visual observation of cell cultures during expansion to eliminate human judgement. The final cell numbers obtained were as in figure 5.33 (next page).

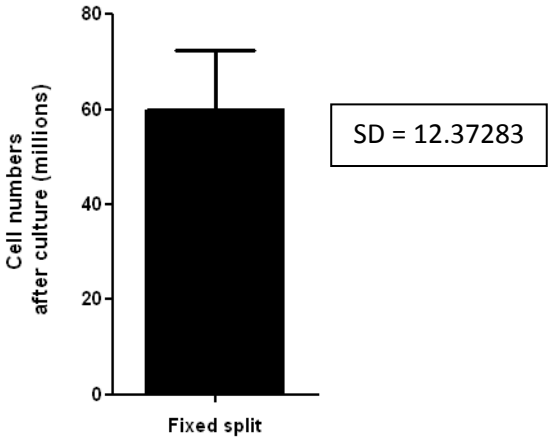


Figure 5.33: Average number of T-cells generated after 18 days of culture using the fixed splitting schedule and EBV peptide stimulation protocol.
EBV specific T-cells generated from fresh blood were cultured in 24-well plates for 18 days using the "fixed splitting schedule" to investigate the average cell number and standard deviation occurring between a different set of donors. n = 7 (this experiment repeated 7 times using fresh blood from 7 different healthy donors).

The standard deviation here in figure 5.33 is much lower at 12.4 compared to 29.7 previously (fig. 5.32). The difference in "mean cell number" between the two methods of expansion is probably due to difference in donors used (59 million with fixed splitting compared to 37 million before).

For comparison these experiments were repeated again with a new set of healthy donors where the T-cells were exposed to both methods of expansion (normal vs. fixed) and final cell numbers and fold expansions were compared figure 5.34 below.

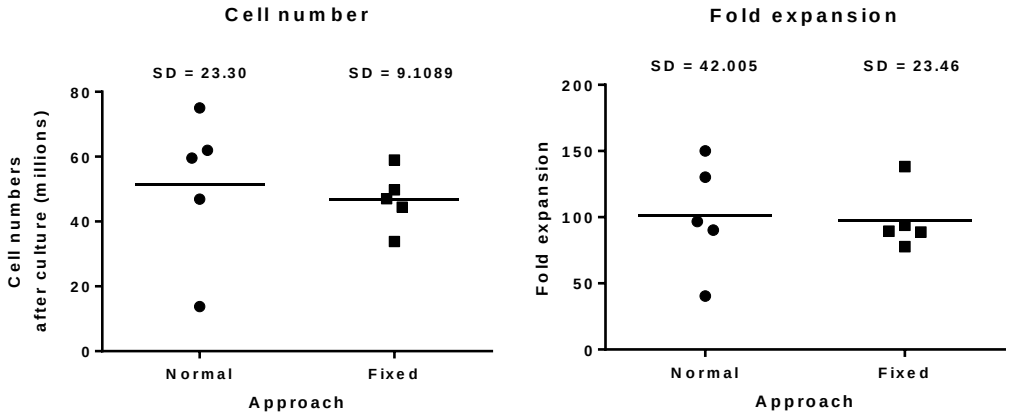


Figure 5.34: Comparison of fixed splitting schedule and normal method of expansion.
The normal (circles) and fixed splitting method (squares) were compared by exposing groups of cells from each donor to either methods of expansion. Culture was carried out for 18 days using EBV specific T-cells generated from fresh blood and the final cell numbers from both methods and standard deviation was calculated along with the fold expansion. n = 5 (this experiment repeated 5 times using fresh blood from 5 different healthy donors).

The variation (standard deviation) in cell number and fold expansion is much lower in T-cells expanded using the fixed splitting schedule (SD = 9 and 23) compared to normal expansion of T-cells (SD = 23 and 42) as in figure 5.34. However, the highest cell numbers were reached using the normal expansion method (75 million) compared to the highest obtained using fixed splitting schedule (58.98 million).

In a separate set of experiments we decided to expand T-cells on rough surfaces using the same fixed splitting schedule and compared it to normal expansion (figure 5.35). The antigen specific T-cells were cultured for 18 days using the EBV peptide stimulation protocol and both methods of expansion were compared.

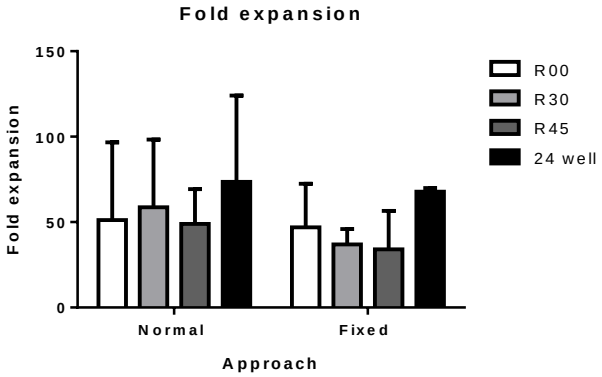


Figure 5.35: T-cells cultured on rough surfaces using fixed splitting schedule.
EBV specific T-cells generated from fresh blood were cultured for 18 days on rough surfaces using both methods of expansion (normal and fixed splitting methods). The material inserts used were: R00 (white), R30 (light grey), R45 (dark grey) and for comparison 24-well plate culture (black). The fold expansion was calculated at end of culture. n = 2 (this experiment repeated 2 times using fresh blood from 2 different healthy donors).

The standard deviation again is much lower in T-cells cultured using the fixed splitting schedule (SD = 2 to 25) compared to the standard deviations in T-cells cultured using the normal method of expansion (SD = 20 to 50).

However, the overall fold expansion is lower in T-cells cultured using fixed splitting schedule. Also R30 is no longer having the best growth (36.9 fold) among the three roughness (R00, R30 and R45) when exposed to the fixed splitting schedule. This is different compared to the normal method of expansion where R30 is 58.7 fold after culture.

2. Peptide pool titrations:

EBV specific T-cells are generated using the EBV peptide stimulation protocol in all our experiments. The peptide concentration recommended to stimulate T-cells is 1µg / ml. The aim of this experiment was to titrate down the concentration of peptide used to stimulate T-cells and find out the lowest concentration sufficient to generate T-cells without loss of functionality or maturation of cells.

Three concentrations of peptide pools were chosen for titration as in Table 5.1.

Table 5.1: Peptide pool concentrations

	µg / ml complete medium		
Concentration	1	0.5	0.1

EBV specific T-cells were generated from healthy donors each exposed to the three concentrations of peptide. The generated cells were cultured separately in 24-well plates for 18 days after which they were harvested and analysed.

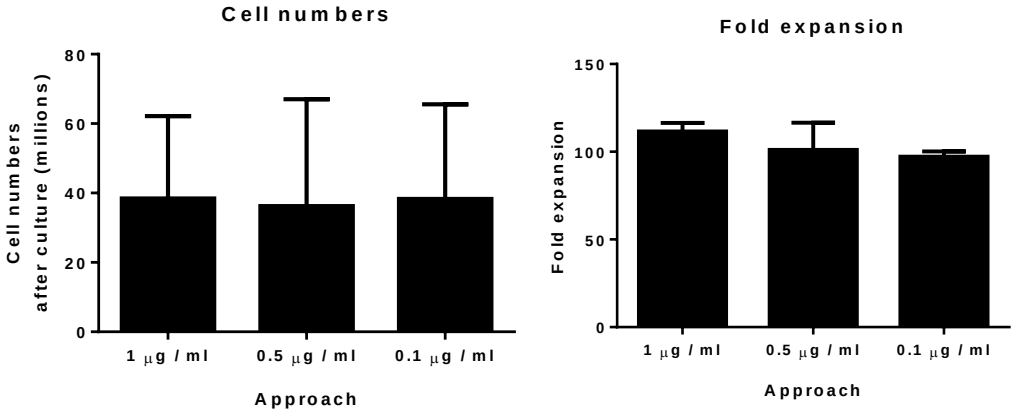


Figure 5.36: T-cell culture for 18 days using three concentrations of peptides.
Three concentrations of EBV peptides were used (1µg/ml, 0.5µg/ml and 0.1µg/ml). The T-cells were generated from buffy coats with these concentrations of peptide using EBV peptide stimulation protocol and cultured for 18 days after which cells were harvested, counted and fold expansion calculated. n = 2 (this experiment repeated 2 times using buffy coats from 2 different healthy donors)

It appears that the fold expansion is quite similar among all three concentrations (figure 5.36) where 1μg/ml reached 111 fold, 0.5μg/ml reached 101 fold and 0.1μg/ml reached 97 fold. The final cell numbers are also comparable as 1μg/ml reached 38 million, 0.5μg/ml reached 36 million and 0.1μg/ml reached 38 million. This indicates that the lowest concentration of peptide may be used for generating T-cells with comparable fold expansion and cell numbers.

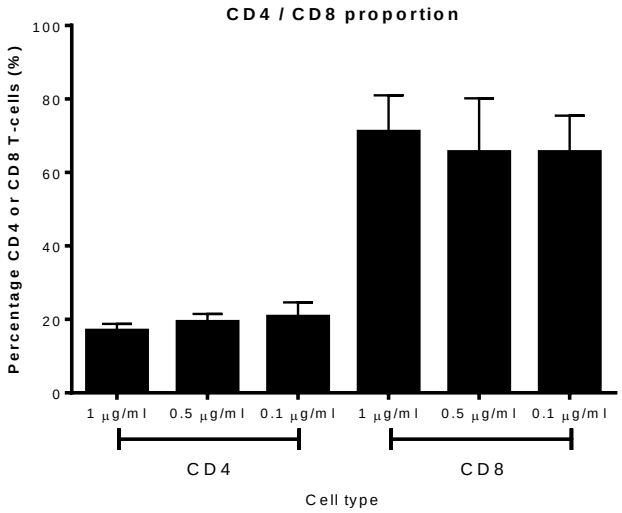


Figure 5.37: CD4 and CD8 proportions after culture for 18 days with three concentrations of peptides.
Three concentrations of EBV peptides were used (1µg/ml, 0.5µg/ml and 0.1µg/ml) and T-cells were generated from buffy coats with these concentrations of peptide using the EBV peptide stimulation protocol. After 18 days growth the cells were stained for CD3, CD4 and CD8 surface markers. The cells were analysed as a percentage of CD3+ T-cells present in test samples. n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors).

Next these T-cells were stained for surface markers CD3, CD4 and CD8 and the proportions analysed. It seems that CD4 and CD8 proportions (which were calculated as a percentage of CD3+ T-cells present in test samples) are at similar levels for the three concentrations of peptide used in our experiment (figure 5.37). In the three concentrations of peptide *(1µg/ml, 0.5µg/ml and 0.1µg/ml)* the percentages of CD4 cells were 16.9, 19 and 20.7% respectively. The percentages of CD8 cells were 71, 65 and 65% respectively. In our measurements roughly 12 to 16% of the cells were negative for CD4 or CD8 and can be attributed to other cell types such as double negative cells[19] and NKT cells[20].

The same EBV specific T-cells were also re-stimulated with 1µg/ml of EBV peptide for 6 hours with Brefeldine-A and stained for cytokine markers (IFNγ and TNFα) and surface marker for degranulation (CD107a). During flowcytometry analysis, live CD4 and CD8 cells that responded to peptide stimulation were analysed (figures 5.38 and 5.39). It appears that there is no difference in IFNγ or TNFα secretion among the cells generated from three peptide concentrations.

In figure 5.38 the proportions of CD4 cells secreting IFNγ were very similar across the three concentrations of peptide tested (3.6% to 4.7%). This was also the same with CD8 cells (6.5% to 7.3%) secreting IFNγ. The number of cells secreting TNFα was also very similar

across the three concentrations where 2.8% to 3.2% of CD4 cells and 5.2% to 6.2% of CD8 cells secreted TNFα (figure 5.39).

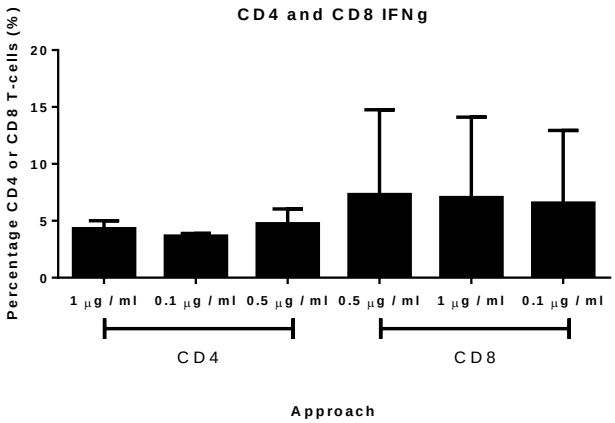


Figure 5.38: Percentage of T-cells secreting IFNγ after 18 days culture with three concentrations of peptides.
EBV specific T-cells generated from buffy coats with the three concentrations of peptide (1µg/ml, 0.5µg/ml and 0.1µg/ml) and culture for 18 days were re-stimulated and stained for surface markers CD4 and CD8 and cytokine marker IFNγ. The proportion of cells responding were analysed by flowcytometry. n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors)

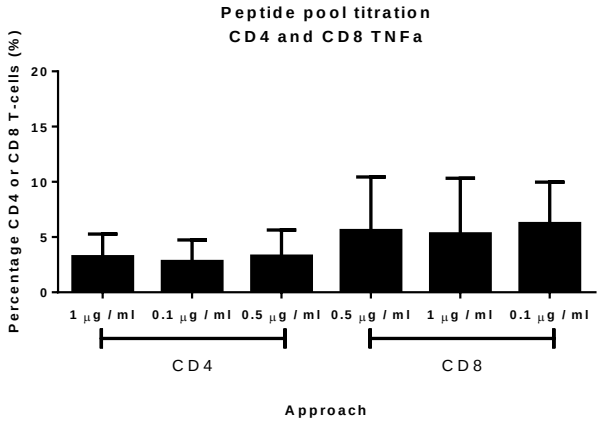


Figure 5.39: T-cell TNFα secretion after 18 days culture with three concentrations of peptides.
EBV specific T-cells generated from buffy coats with the three concentrations of peptide (1µg/ml, 0.5µg/ml and 0.1µg/ml) and culture for 18 days were re-stimulated and stained for surface markers CD4 and CD8 and cytokine marker TNFα. The proportion of cells responding were analysed by flowcytometry. n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors)

Analysis of CD107a expression after re-stimulating T-cells with 1µg/ml EBV peptide showed similar levels in both CD4 and CD8 cells across the three concentrations of peptides investigated (figure 5.40). On average around 58 to 60% of CD4 cells expressed CD107a which was similar to CD8 cells (58 to 61%). These results along with cytokine secretion together suggest that T-cell functionality is not affected by lower concentrations of peptide used to generate T-cells.

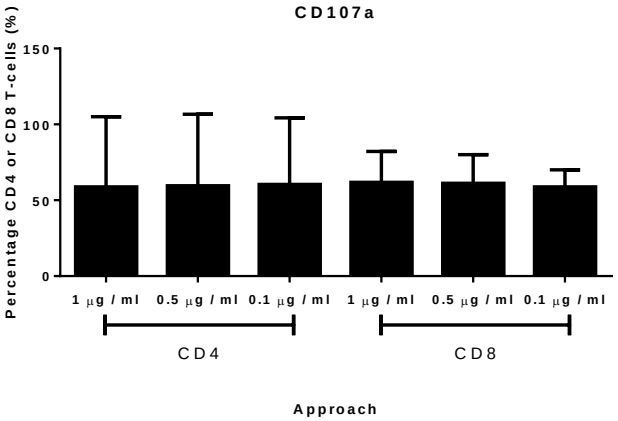


Figure 5.40: Expression of CD107a in T-cells after 18 days culture with 3 concentrations of peptides.
EBV specific T-cells generated from buffy coats with the three concentrations of peptide (1µg/ml, 0.5µg/ml and 0.1µg/ml) and culture for 18 days were re-stimulated and stained for surface markers CD4, CD8 and CD107a. The proportion of cells responding were analysed by flowcytometry.
n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors)

The cells were also stained for markers of maturation (CD45RA and CCR7). T-cell maturation was classified as: Effector memory (CD45RA- and CCR7-), Central memory (CD45RA- and CCR7+) and Effectors (CD45RA+ and CCR7+).

The maturation states of CD4 and CD8 T-cells are similar across the three concentrations of peptide investigated (figure 5.41). Around 27 to 28% of CD4 cells were effector cells and 26 to 27% were central memory while 32 to 33.7% were effector memory. Among CD8 cells 60 to 65% were effector cells, 2.5 to 2.7% were central memory and 26 to 30.8% were effector memory.

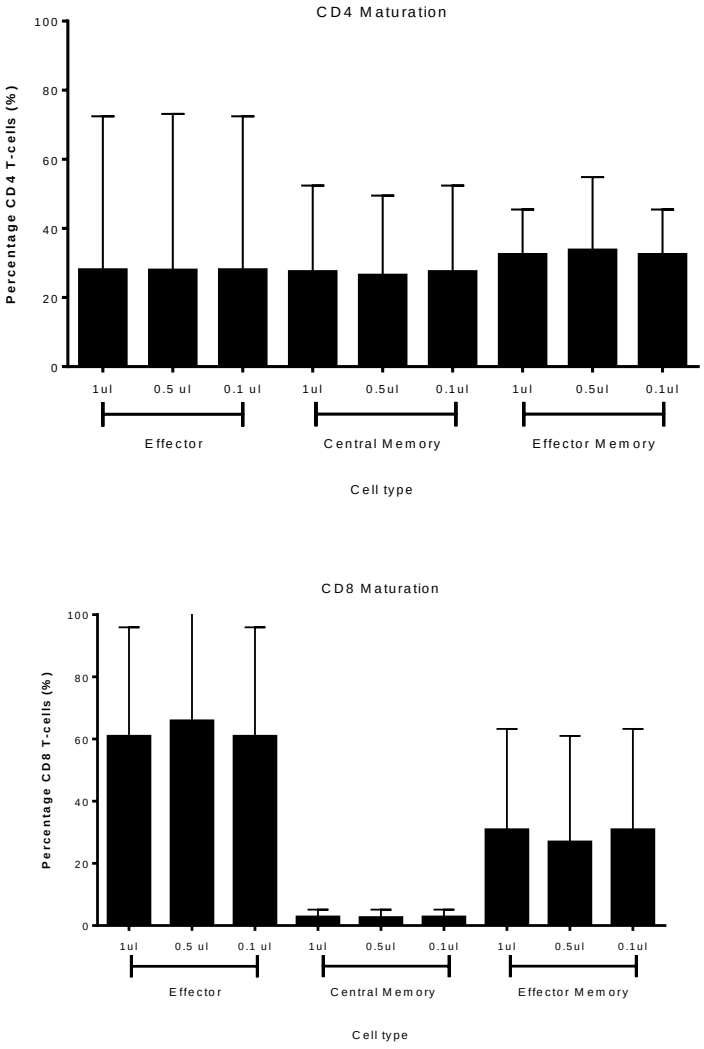


Figure 5.41: Maturation states of CD4 and CD8 cells after 18 days culture with three concentrations of peptides.

EBV specific T-cells generated from buffy coats and cultured with three concentrations of peptide were stained for markers of maturation (CD45RA and CCR7) and surface markers (CD4 and CD8) after harvest on day 18. In the figure effector memory are CD45RA- and CCR7-, central memory are CD45RA- and CCR7+ and effectors are CD45RA+ and CCR7+. n = 3 (this experiment repeated 3 times using buffy coats from 3 different healthy donors)

Section 4:

T-cell manipulation during inoculation, culture and harvesting

(Cell culture dissociation experiments)

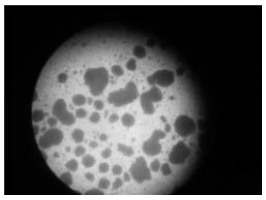
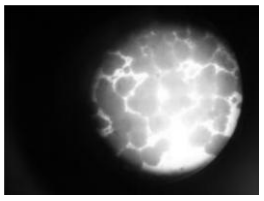
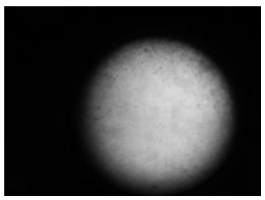
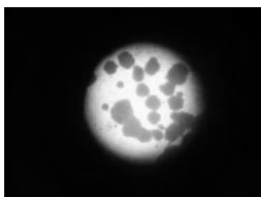
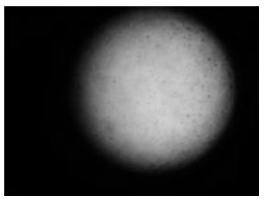
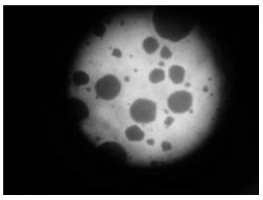
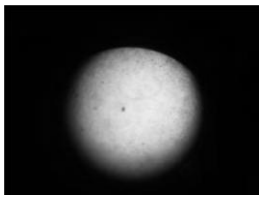
T-cell manipulation during inoculation, culture and harvesting

(Cell culture dissociation experiments)

The aim of these experiments was to develop a technique to manipulate T-cells within a closed culture system during the stages of inoculation, culture and harvesting. This should be done while maintaining the closed nature of the culture system to ensure sterile conditions. T-cell manipulation involves breaking cell clumps and homogenization of the medium and is required mainly at three stages of culture: Inoculation, expansion and harvesting. The ideal way would be to manipulate T-cells so that they are not physically damaged during the process and it should be efficient enough to be used without opening the culture system.

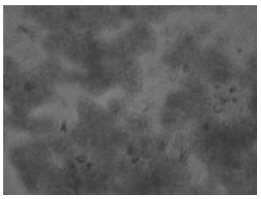
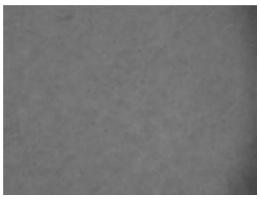
It was decided that a mixing-table (please see “mixing-table” in “methods” page 118) would serve our purposes and a set of experiments were performed. T-cells were expanded until large clumps could be seen in cultures using 24-well plates. These were then exposed to the mixing-table at various speeds and time points Table 5.2. For comparison cell clumps were manually broken using pasture pipettes which is the normal method used in our labs during T-cell expansion.

Table 5.2: Breaking of T-cell clumps in a 24-well plate using the mixing-table and pasture pipette.

Experiment	Speed (rpm)	Time (mins)		
		0	5	10
1	300	No effect		
2	500			
3	700			← after 1 min
4	Pipette (manual)			← after 3 mins

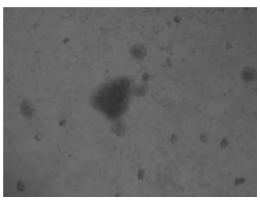
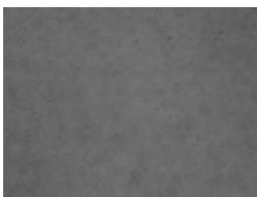
Machine speeds at 300 revolutions per minute (rpm) were not able to dissociate cell clumps in T-cell cultures in 24-well plates. Higher speeds of 500 and 700 rpm were able to do so in 10 minutes and 1 minute respectively. For comparison manual dissociation of cell clumps using pasture pipettes was possible within 3 minutes. The same experiment was repeated again using 12-well plates with the mixing-table and the results are shown in Table 5.3.

Table 5.3: Breaking of T-cell clumps in a 12-well plate using the mixing-table.

Experiment	Speed (rpm)	Time (mins)		
		0	5	7
1	300	No effect		
2	500		No effect	

A speed of 300 rpm produced no visible results except for the clumping of cells at the centre around 10 min (not shown). It took 7 minutes at 500 rpm to induce dissociation of cell clumps in 12-well plates having T-cell cultures. Higher speeds induced splashing and spilling of medium due to the larger size and higher volume of the wells (compared to 24-well plates) so it was not possible to test this. When repeating the same experiment using 6-well plates the following results were obtained in Table 5.4.

Table 5.4: Breaking of T-cell clumps in a 6-well plate using the mixing-table.

Experiment	Speed (rpm)	Time (mins)	
		0	5
1	300		

A low speed of 300 rpm for 5 minutes was enough to break all cell clumps seen in culture and higher speeds induced splashing and spilling hence, testing was not possible.

Section 5:

Comparison of existing systems

Comparison of existing systems

The aim of this experiment was to compare culture systems that could be used to culture antigen specific T-cells. For comparison the regular 24-well plate was used and expansion was carried out in two ways: normal expansion and expansion using the fixed splitting schedule.

Below were the approaches used in this experiment:

	System	Expansion
1	G-Rex bioreactor*	According to manufacturer's instructions
2	Perfusion bioreactor*	According to manufacturer's instructions
3	24-well plate	Fixed splitting schedule
4	24-well plate	Normal expansion

**Please see "G-Rex bioreactor" and "Perfusion bioreactor" in the "methods" section, pages 118 and 119 respectively)*

EBV specific T-cells were generated using the EBV peptide stimulation protocol and expanded for 18 days in the four systems listed above and then cells were counted and analysed.

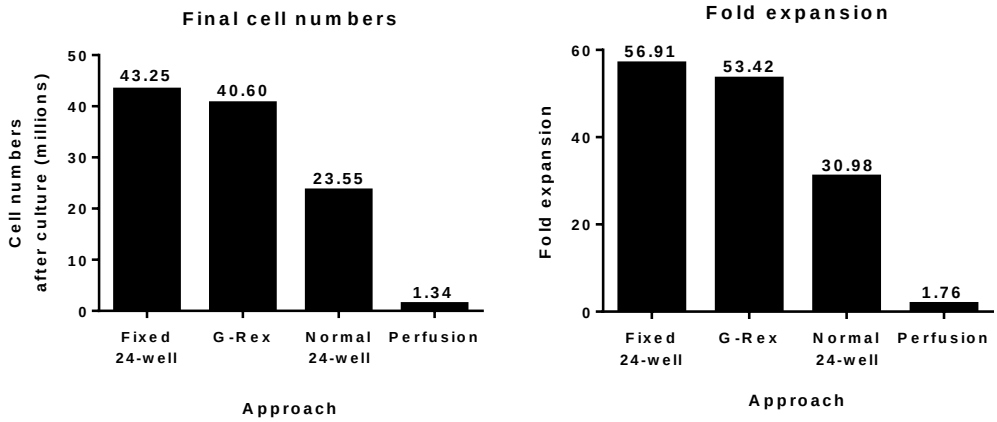


Figure 5.42: T-cell fold expansion and cell number after culture for 18 days in the four systems.
EBV specific T-cells generated from a buffy coat using the EBV peptide stimulation protocol were cultured in the four systems: G-Rex bioreactor, Perfusion bioreactor and 24-well plates (normal and fixed splitting expansion). After 18 days the cells were counted and fold expansion calculated. n = 1 (experiment done once with a single donor)

The highest growth was seen in T-cells cultured using the fixed splitting schedule in 24-well plates and the G-Rex system reaching 43.25 million and 40.6 million respectively. The lowest growth was observed with the perfusion bioreactor which was able to reach only 1.34 million cells after 18 days of culture. On investigating the CD4 and CD8 proportions before and after culture in the four systems, the following results in figure 5.43 were obtained.

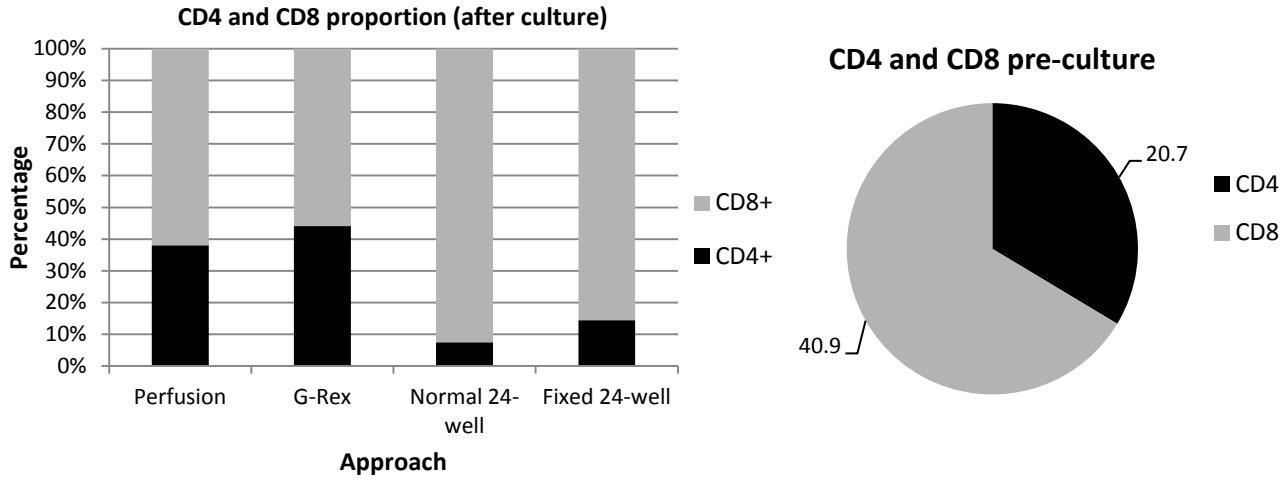


Figure 5.43: CD4 and CD8 proportions before and after culture for 18 days in the four systems.
EBV specific T-cells generated from a buffy coat were cultured in the four systems: G-Rex bioreactor, Perfusion bioreactor and 24-well plates (normal and fixed splitting expansion). The cells were stained for CD4 and CD8 markers before and after 18 days of culture. n = 1 (experiment done once with a single donor)

The proportions of CD4 and CD8 cells before culture was roughly 1:2 and this ratio was maintained in the G-Rex and Perfusion systems, while a lower proportion of CD4 cells is seen in the 24-well plate cultures (both fixed splitting schedule and normal expansion).

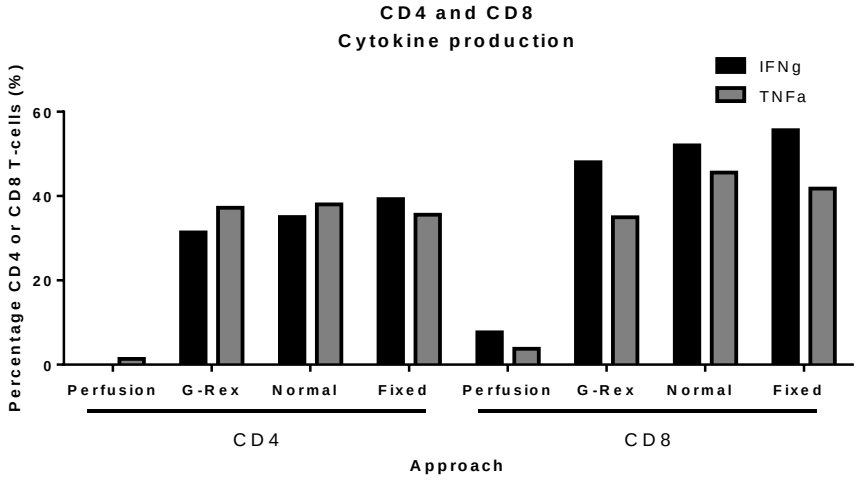


Figure 5.44: Cytokine secretion in T-cells cultured for 18 days in the four systems.
The EBV specific T-cells generated from a buffy coat and cultured in the four systems: G-Rex bioreactor, Perfusion bioreactor and 24-well plates (normal and fixed splitting expansion) for 18 days were re-stimulated with peptides and stained for surface markers CD4 and CD8 and cytokine markers IFNγ and TNFα.
n = 1 (experiment done once with a single donor)

The EBV specific T-cells from the four approaches were re-stimulated with EBV peptides for 6 hours with Brefeldine- A and then stained for cytokines (IFNγ and TNFα). The percentage of cells secreting IFNγ upon re-stimulation was similar in the G-Rex system (31% CD4, 48% CD8), 24-well normal (35% CD4, 52% CD8) and 24-well fixed (39% CD4, 55.6% CD8) cultures. However, only 7.6% of CD8 cells in

the Perfusion bioreactor secreted IFNγ while it was not possible to measure IFNγ secretion in CD4 cells grown in the perfusion bioreactor since the readings were too low to be analysed accurately.

TNFα secretion followed a similar pattern too where 37% CD4 cells and 34.9% CD8 cells in G-Rex while only 1.4% CD4 cells and 3.8% CD8 cells in the perfusion bioreactor secreted TNFα. The 24-well plate cultures had similar levels in both methods of expansion where 38% CD4 cells and 45.5% CD8 cells (normal expansion) and 35.5% CD4 cells and 41.7% CD8 cells (fixed split schedule) secreted TNFα.

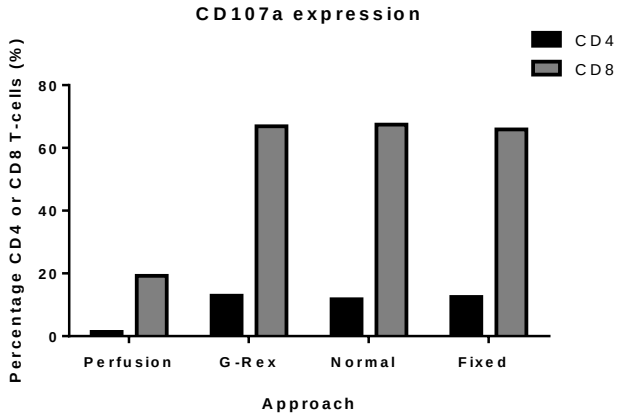


Figure 5.45: CD107a expression in T-cells cultured for 18 days in the four systems.
The EBV specific T-cells generated from a buffy coat and cultured in the four systems: G-Rex bioreactor, Perfusion bioreactor and 24-well plates (normal and fixed splitting expansion) for 18 days were re-stimulated with peptides and stained for surface markers CD4 and CD8 and cytokine markers IFNγ and TNFα.
n = 1 (experiment done once with a single donor)

The same T-cells were also stained for marker of degranulation (CD107a) and the expression of CD107a was similar in the G-Rex and 24-well plates (normal / fixed) while the Perfusion system showed very low levels of CD107a expression (figure 5.45).

The G-Rex, 24-well (normal) and 24-well (fixed) demonstrated 12.9%, 11.75% and 12.52 % CD4 cells expressing CD107a respectively while only 1.4% CD4 cells in the perfusion bioreactor were found to express CD107a. A higher percentage of CD8 cells expressed CD107a in G-Rex (66.8%), 24-well normal (67.46%) and 24-well fixed (65.92%) but a lower percentage was seen in the perfusion bioreactor (19.2%).

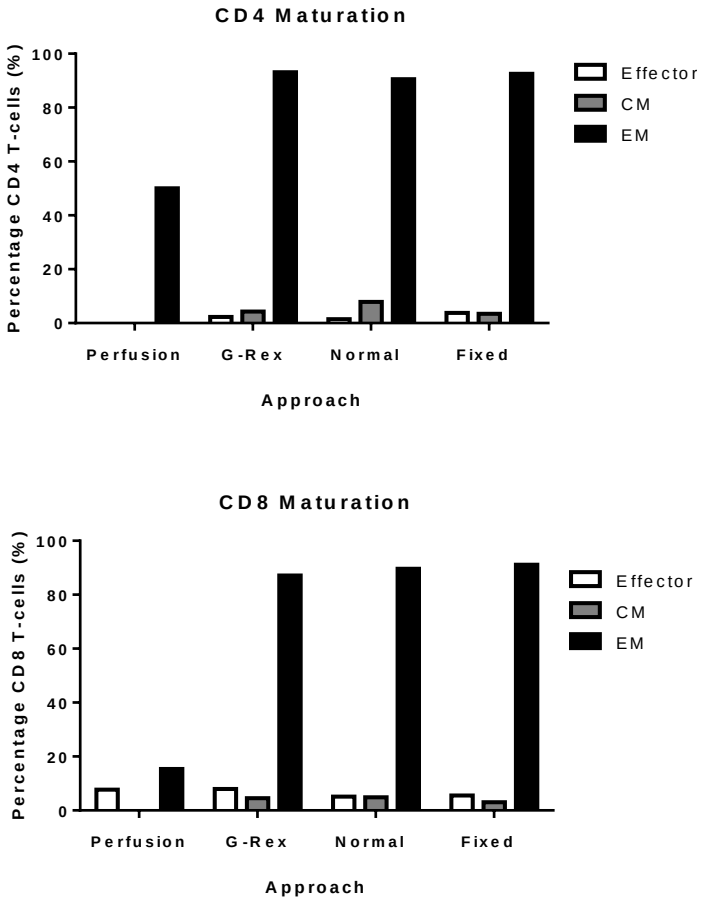


Figure 5.46: Maturation states of T-cells cultured for 18 days in the four systems.
EBV specific T-cells generated from a buffy coat and cultured in the four systems: G-Rex bioreactor, Perfusion bioreactor and 24-well plates (normal and fixed splitting expansion) for 18 days were stained for markers of maturation (CD45RA and CCR7) and surface markers (CD4 and CD8) after harvest on day 18. In the figure effector memory are CD45RA- and CCR7- (black bars), central memory areCD45RA- and CCR7+ (grey bars) and effectors are CD45RA+ and CCR7+ (white bars). n = 1 (experiment done once with a single donor)

Next the T-cells were stained for markers of maturation (CD45RA and CCR7). T-cell maturation was classified as: Effector memory (CD45RA- and CCR7-), Central memory (CD45RA- and CCR7+) and Effectors (CD45RA+ and CCR7+). T-cells showed similar maturation patterns when cultured in the G-Rex system and 24-well plates (normal / fixed) among both CD4 and CD8 cells (figure 5.46).

Effector memory cells were the largest proportion of cells in the G-Rex (93% CD4, 87% CD8), 24-well normal (90.5% CD4, 89.6% CD8) and 24-well fixed (92% CD4, 91% CD8). There were a smaller proportion of effector memory cells in the perfusion bioreactor (50% CD4, 15.4% CD8) compared to other approaches.

The other cell types (central memory and effector cells) were between 3 to 7% across all the approaches except for the perfusion bioreactor which expressed mostly an effector memory phenotype. The central memory and effector cells had a cell count that was too low to analyse accurately in the perfusion bioreactor.

Discussion

Discussion

In this study we investigated the interactions between T-cells and the culture system which provided an insight into factors that may affect T-cell growth in our current culture systems. This knowledge helps us in designing better systems for T lymphocyte culture with minimal influence on T-cell function, proliferation and phenotype.

The need for investigating these interactions are as described previously in the introduction (page 11), that we do not know how the culture system can influence T-cell growth in terms of functionality, proliferation and phenotype. We have been using the 24-well plate culture system for a long time and there is very less information about how the T-cells interact with the culture system that they are grown in. A possible use for this kind of information is when constructing a culture system different to the 24-well plate system and maintaining T-cell functionality that is appropriate for immunotherapeutic applications.

Interactions between T lymphocytes and the culture chamber

The interactions mentioned in this study are not exhaustive, while there is a high possibility of other factors playing a role during T-cell expansion which were not investigated due to the limited scope of this investigation, such as: surface treatment (hydrophilic or hydrophobic), protein adsoption (on a material surface), material degradation or digestion (due to cellular action) and effect of shape on cell growth (only circular chambers used in our experiments).

The factors that were eventually selected for investigation in our study were obvious ones that had a high likelihood to influence T-cell culture. They were also very manageable and could be studied with minimal resources and in an accurate scientific manner that is reproducible. The figure below is repeated from page 13 in “Experimental design”:

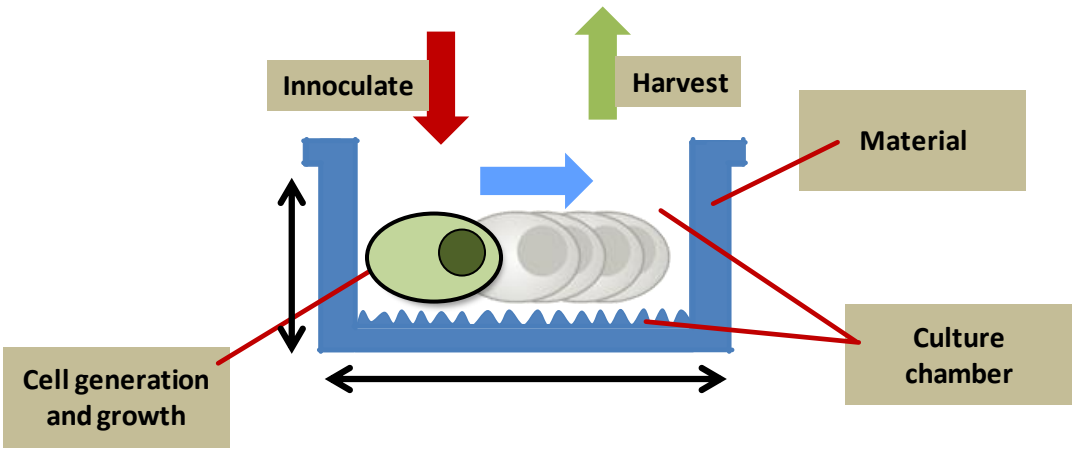


Figure 6.1: T lymphocyte interactions during culture.

We also opted to focus on closed systems rather than open ones due to issues with cross-contamination and sterility during T-cell culture. The nature of a closed culture system necessitates avoiding direct physical interaction with the contents inside the system to prevent contamination. Visual perception of the culture process may also be limited by the transparency of the walls and construction geometry of the system. This makes it very difficult when handling T-cells in such a system since the normal culture process in 24-well plates requires a lot of manipulation by the observer for the entire period.

We focused on techniques that could be used when culturing T-cells in such a closed system without the need for direct manipulation of the contents. Also standardizing the culture process was attempted in the experiments dealing with: "cell generation and growth" and "manipulation during inoculation, culture and harvesting".

This table is a reminder of the experiments done which is also in page 15 in "Experimental design":

Table 6.1: Experiments done to investigate T cell interactions.

T-cell interactions	Experiments
Materials	Material testing (R00) –PS,PEI,PEU,PSAN,PC
	Material testing (R30) –PS,PEI,PEU,PSAN,PC
	Material testing (R45) –PS,PEI,PEU,PSAN,PC
	Material induced T-cell function experiment
Culture chamber (shape, size, volume and culture surface)	Geometry testing
	Roughness testing experiments (R00, R30 , R45)
	Electro-spun materials
Cell generation and growth (Cost and end product)	Cell number experiments
	Fixed splitting experiments
	Peptide pool titration experiments
Manipulation during Inoculation, culture and harvesting	Cell culture dissociation experiments
Comparison of existing systems [2]	System comparison experiments

[2] *In addition two existing systems were also investigated to find out additional influences to T-cell growth which is unique to such systems.*

Section 1:

T-cell material interactions

T-cell material interactions

The material used to construct the culture chamber can have a major influence on T-cell growth since it is in contact with the cell for the longest period during cell growth. Ideally, the culture surface material should not adversely affect functionality in antigen specific T-cells as this would be undesirable if they are to be used later for immunotherapy. While several studies have investigated the influence of material on T-cells for short periods of time (<6 days of culture) [21-24], there is currently no information on the effects of prolonged contact with a particular material. Additionally, the use of material "fragments" for testing might pose several problems as in figure 6.2.

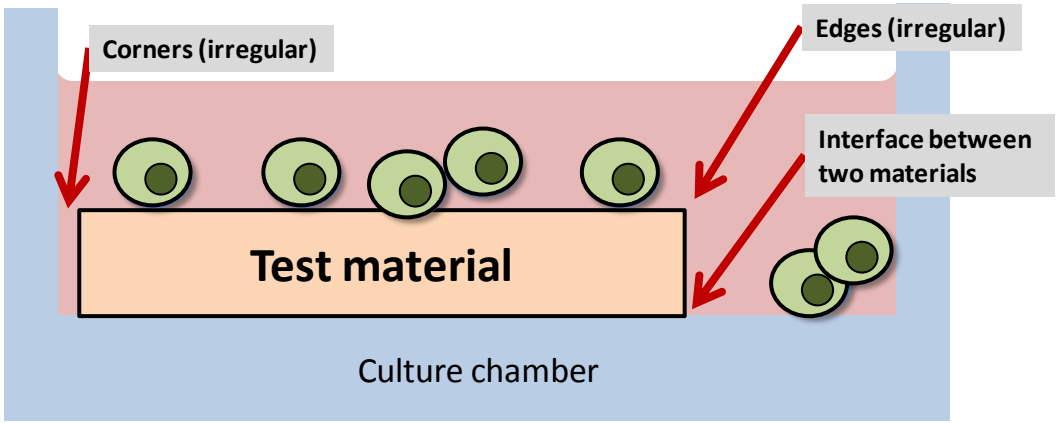


Figure 6.2: Unwanted influences on T-cell growth during material testing experiments.
T-cells are semi-adherent and tend to change position when fragments of test material are introduced into the culture chamber.

T lymphocytes are semi-adherent in nature and tend to move about when turbulence is introduced in the culture system (e.g. via a pasture pipette). This makes it troublesome during material testing experiments and can give rise to several problems as mentioned in figure 6.2: Firstly, since the material fragment will be placed on the bottom of the culture chamber there is an "interface" between two materials (test and culture chamber material). This means T-cells that aggregate in these corners will be exposed to two kinds of material during the experiment. So the direct influence of test material on T-cell functionality and growth cannot be accurately assessed.

Secondly there is also the influence of corners and edges of the test material as most T-cells are known to aggregate in such corners. The test material fragment in combination with the culture surface can form niches where T-cells can collect or get damaged on movement of the test material. Also since the exact position of the test material cannot be reproduced accurately for every experiment, this proves disadvantageous as it generates another variable in our investigation that could influence T-cell growth.

Material cup inserts have fixed dimensions, are reproducible and culture surfaces can be modified

When considering the drawbacks of using a set-up as described in figure 6.2, a different "solution" was proposed in our group. The material cup inserts described in "methods", figure 8.5 and 8.6 on page 113 was used for testing materials. All material cup inserts are generated via injection moulding which is capable of generating inserts with the same dimensions for all types of materials tested. The cells are surrounded by only the test material making it more accurate to study the effects of the material on T-cell growth, function and phenotype. There is no risk of damage to the cells as there are no moving parts and the geometry of the cup can be reproduced for each and every experiment. This includes surface roughness which was generated with the use of a stamp during the injection moulding process ensuring the same rough texture was used for all experiments. Three grades of roughness were employed; R00, R30 and R45, please see "methods" in page 115.

Tecoflex (TFX) has the poorest growth while other four materials are similar in fold expansion

Fresh blood was used to extract T-cells and pan T-cell stimulation was carried out before plating in the five materials. T Lymphocyte growth on the four materials: Polystyrene (PS), Poly(styrene-co-acrylonitrile) (PSAN), Poly(etherimide) (PEI) and Polycarbonate (PC) have almost similar growth patterns in all three grades of roughness (R00, R30 and R45) as seen in figures 5.3, 5.5 and 5.7 pages (20, 22 and 24). However, Tecoflex (TFX) has the lowest growth irrespective of the grade of surface roughness used in all three experiments (figures 5.3, 5.5 and 5.7).

Another interesting finding is that in the first experiment (figure 5.3) the 24-well plate culture has a slightly higher fold expansion compared to the material cup inserts despite all approaches having the same starting cell number (0.5 million cells). The reasons for this could be two: firstly, the 24-well plates are all surface treated to be hydrophilic via oxygen plasma treatment (done commercially by the manufacturer) and secondly, the culture chamber size (geometry) of the wells in a 24-well plate are much larger than the material cup inserts. This might give T-cells in the 24-well plates an advantage as there is more medium and space available for expansion due to the larger geometry. Also hydrophilic surfaces are known to be more blood-compatible since they resemble the blood vessel surface in terms of its hydrophilic nature [25-27].

One of the limitations of this kind of set-up is that the 24-well plate cannot be used as a "control" due to the above mentioned factors namely: Size of the chamber and surface treatment. During culture the volume possible in a single well in a 24-well plate is approximately 2 ml compared to a material cup insert (approx. 1 ml). This means during medium replacement, the cells in a 24-well plate culture are exposed to double the amount of new medium compared to what is possible in material cup inserts. Then there is also the surface treatment which provides a better surface for attachment. These variables are not controllable and hence we cannot use the 24-well plate as a control in our studies despite the fact that it is the most common culture system used in T-cell culture. The 24-well plate cultures were used just to provide a comparison to the material cups.

Also at the time of the experiment it was not possible to apply the same kind of hydrophilic surface treatment to the material cup inserts as this required special equipment which was not available to us. In the future, repetition of this experiment can be attempted if surface treated cups become available. This is to take account of the influence hydrophilic surfaces have on T-cell growth. Additionally, the effect of contact angle of surfaces (hydrophilic/hydrophobic balance of a material) on T-cell growth would also provide more information on T-cell material interaction in culture.

Culture surface roughness has an influence on T-cell growth.

In the second experiment (figure 5.5) material cup inserts with a roughness grade of "R30" were used. These cups were manufactured by using a stamp during the injection moulding process. The pattern of roughness was uniform across all cups which carried the label of "R30" roughness.

The T-cell expansion in the R30 material cups matched the expansion in the 24-well plate culture. This pattern of growth is in contrast to what was seen in figure 5.3 (first experiment with R00) where the cups showed a lower growth compared to 24-well plate cultures.

It appears that roughness has a positive influence on T-cell growth in the material cups. Where despite having a smaller volume and no surface treatment the growth in the end was almost equal if not more than the 24-well plate growth.

When this experiment was repeated with a higher grade of roughness, "R45" (figure 5.7), the fold expansion diminished and resembled that of T-cells in the first experiment (figure 5.3) where the expansion in the 24-well plate culture was higher than the cups. This is interesting since it suggests an optimum grade of roughness is necessary to produce the best T-cell expansion.

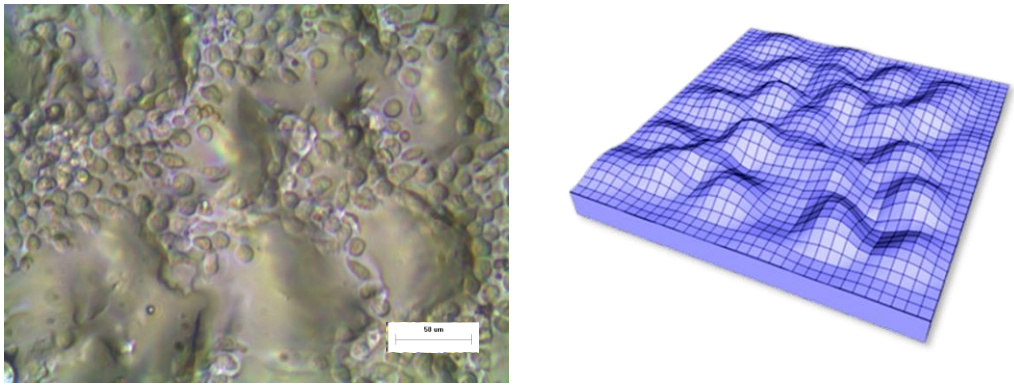


Figure 6.3: T-cell growth on a rough surface (R30) and schematic of a rough surface (R30)
Scale is 50 µm in image

The starting cell numbers in the three material testing experiments with roughness grades R00, R30 and R45 were the same (0.5 million cells) and all were exposed to the same conditions (stimulation method, medium, CO_2, temperature and IL-2 concentration). The different fold expansions observed were solely due to the roughness of the material cups themselves. This finding could be attributed to the fact that T-cells in nature are also exposed to "wavy" or "irregular" surfaces such as those found in lymph nodes, thymus and bone marrow where there are lots of places for cells to collect, aggregate and form niches [28]. Perhaps it is possible that T-cells tend to prefer "corners" rather than "plain surfaces" during growth since they provide "niches" similar to those found in nature.

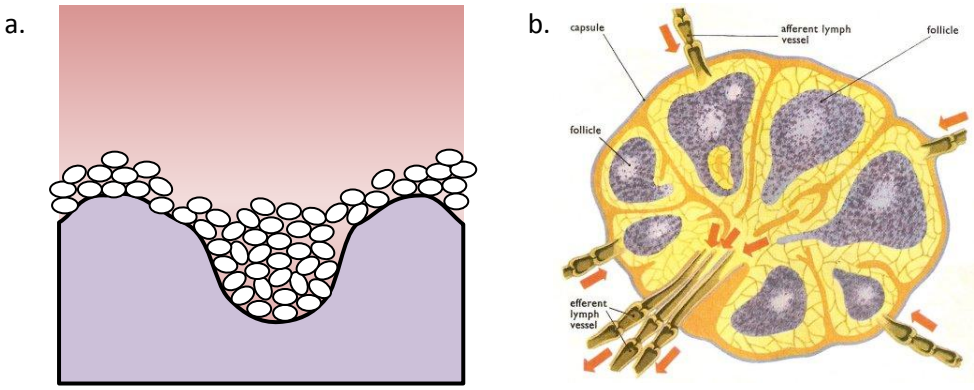


Figure 6.4: T-cells aggregate (a) in depressions on rough surfaces (schematic) and (b) in follicles present in lymph nodes. *(figure b was taken from the website: http://www.daviddarling.info/encyclopedia/L/lymph_node.html on the 9th February 2013)*

Hence, the use of rough surfaces for T-cell culture (figure 6.3) is probably more close to the natural conditions of a T-cell than what a flat surface can provide (figure 6.4). The frequency of cell-to-cell contact needed for growth and antigen presentation is facilitated and probably higher when T-cells

are grown on rough surfaces. One can assume that the diffusion of cytokines and other growth factors to surrounding cells is also better on rough surfaces since more cells are closer to each other when gathered in niches than they would be on a plain surface. These findings were the basis of the "roughness testing" experiments which are described in the next section "T-cell culture chamber interactions" on page 81.

Hierarchical influence of culture surface components on T-cell culture

The three components of the culture surface investigated so far: material, roughness and culture chamber size appear to have an effect on T-cell growth in varying degrees. There seems to be a hierarchy of influence on T-cell growth where the highest influence is exerted by the material itself. This is apparent in the case of TFX where irrespective of the roughness there is very poor growth figures 5.3, 5.5 and 5.7 pages (20, 22 and 24). Even judging from the appearance of T-cells on the day of harvest, there are much smaller clumps (figure 5.4) and much fewer cells (figures 5.6 and 5.8) seen with cultures on TFX compared to the other four materials. This indicates a prominent role of the material influence on cell growth as these cells are in constant contact throughout the culture period.

The next is the surface roughness, as mentioned earlier where despite having a smaller volumes and no surface treatment the fold expansion is similar to the 24-well plate culture which has a larger geometry and surface treatment (figure 5.5) but this occurs only on roughness grade R30.

Lastly the size of the culture chamber seems to influence cell growth where despite having the same starting cell numbers there was a higher expansion in the "larger" 24-well plate compared to the material cups in the first experiment (figure 5.3). Perhaps the higher medium volume and surface treatment in 24-well plates are responsible for the slightly higher growth seen in 24-well plate cultures.

T-cells appear to be "over activated" when cultured on Tecoflex (TFX)

T Lymphocytes were generated using the EBV peptide stimulation protocol and after 18 days of culture they were stained for activation markers without re-stimulation and analysed as in figure 5.9, page 26.

T-cells cultured on TFX during the material testing experiments demonstrated very poor cell growth irrespective of the grade of roughness.

The results show quite a large proportion of T-cells (CD3+) express TNFα and CD107a when cultured on the material TFX irrespective of the roughness. This is not seen with T-cells grown on the other materials in three grades of roughness (R00, R30 and R45).

It is possible that this might be as a result of over activation of T-cells when in contact with TFX material and perhaps this in turn after a prolonged period could induce cell exhaustion and cell death. This being the case, it makes TFX an unsuitable material due to the fact that cells need to be functional at the end of culture in order to be used in immunotherapy. Clearly the material is having an influence on the cell growth and activation status.

Our primary aim was to identify materials that are suitable for T-cell growth which do not influence T-cell function, phenotype or expansion. In this study we did not investigate the exact cause of T-cell "over activation" after exposure to TFX material. Studies involving Left Ventricular Assist Devices (LVADs) have shown that T-cells in contact with polyurethane based materials undergo activation induced cell death (AICD) [29]. An increase in Calcineurin activation and nuclear translocation of NFATc was found to cause apoptosis in CD4 T-cells when exposed to polyurethanes [30]. This was also shown to cause T-cell dependant B-cell activation via a CD40-CD40 ligand interaction [31]. Alternatively, polyurethane/calcium phosphate composites have been safely used as weight-bearing orthopaedic implants showing no adverse inflammatory response in rats [32].

As an idea for further studies, it would be interesting to identify the exact chemical groups responsible for activation in T-cells when in contact with polyurethanes. Perhaps oxygen plasma treated polyurethanes might prove to be more compatible for T-cell growth.

T-cell functionality and choosing the right material

The functionality of T-cells can be assessed in terms of cytokine secretion (IFNγ, TNFα) and degranulation (surface expression of CD107a) in response to stimulation by EBV peptides [33-35]. The EBV peptide stimulation protocol was used to generate antigen specific T-cells which were cultured on four materials (TFX was not included in this experiment since it was shown to be unsuitable for T-cell culture from the previous experiments) and after culture for 18 days the cells were harvested and analysed for functionality by re-stimulation with EBV peptide.

As a negative control DMSO was used instead of EBV peptides and as positive controls T-cells were stimulated with a mixture of phorbol 12-myristate 13- acetate (PMA) and Ionomycin. Readings from the two controls are not shown but were used when analysing readings from EBV peptide stimulation.

The functionality of the T-cells seems to be unaffected by the four materials used in this experiment due to the similar levels of cytokine secretion and CD107a expression among the four materials tested (figures 5.11, 5.12 and 5.13, pages 29, 30 and 31 respectively).

Also there appears to be no change in the CD4 and CD8 proportions before and after culture on the four materials tested figure 5.14. These findings are relevant when choosing a material to construct a bioreactor for T-cells which are intended for Immunotherapy. The ideal system should not influence the proportions of CD4 and CD8 cells or their functionality. This is extremely important since both of these populations are needed when it comes to mounting an immune response against a pathogen [36-38]. It has been found in mice, that CD4 cells are required for maintaining long term immunity against a pathogen while the CD8 cells are responsible for clearing up infections [39]. This is also true in the case of humans where in the absence of CD4 it is not possible to mount an effective immune response with CD8 cells alone [40].

Even though TFX was not included in this experiment, we did look at the CD4 and CD8 proportions after culture on TFX and it was found to be affected (please see appendix figure 1). The proportion of CD4 cells was low in TFX compared to the other materials which is another point against the use of TFX to culture T-cells.

There is however, an interesting finding with the T-cells grown in 24-well plates which show a higher fold expansion compared to the material cup inserts (figure 5.10). This is interesting since geometry was found to play the smallest role in the previous experiments dealing with culture for 8 days. It appears that geometry of the culture chamber plays a major role over prolonged culture (18 days). These findings also correlate with Geometry testing experiments in the next section (page 77).

Section 2:

T-cell culture chamber interactions

T-cell culture chamber interactions

Once it was clear which kind of materials may be used for culturing T-cells the next obvious factor to investigate was the influence of the culture chamber on T-cell growth. Since T-cell numbers are increasing during culture, the affects of the culture chamber could be divided into two phases: The initial phase which is immediately after starting the culture until day 5 or 6 when cells have started to proliferate (please see appendix figure 2) and the later phase during which exponential proliferation takes place and this depends on the blood donor.

Since T-cells are very few in number at the beginning of culture, they tend to be easily influenced by the increased space between cells which can drastically affect diffusion of cytokine and cell-to-cell interaction needed to maintain healthy populations of T-cells [41-44]. T-cell survival depends on appropriate stimulation between cells which is in turn dependent on the geometry (shape and size) of the culture chamber itself. Once distance between cells reduce, the efficiency of cell to cell communication is very high and is then less dependent on inter-cellular space [45]. At later stages during culture the cell numbers should have increased sufficiently to be more resistant to any changes in culture chamber geometry.

Smaller geometries are better for T-cell growth during the initial stages of culture and larger ones during later stages (days 6 or 7 onwards)

EBV specific T lymphocytes were generated using the EBV peptide stimulation protocol and were cultured in the four geometry approaches (please see: "*Geometry testing approaches*" in "*methods*" page 111). The approaches mentioned here were designed so that gradual and sudden changes in geometry during the initial stages of culture can be investigated if they have an influence on cell growth. The culture in the four approaches was carried out for 18 days and the fold expansion was calculated as in figure 5.16, page 35.

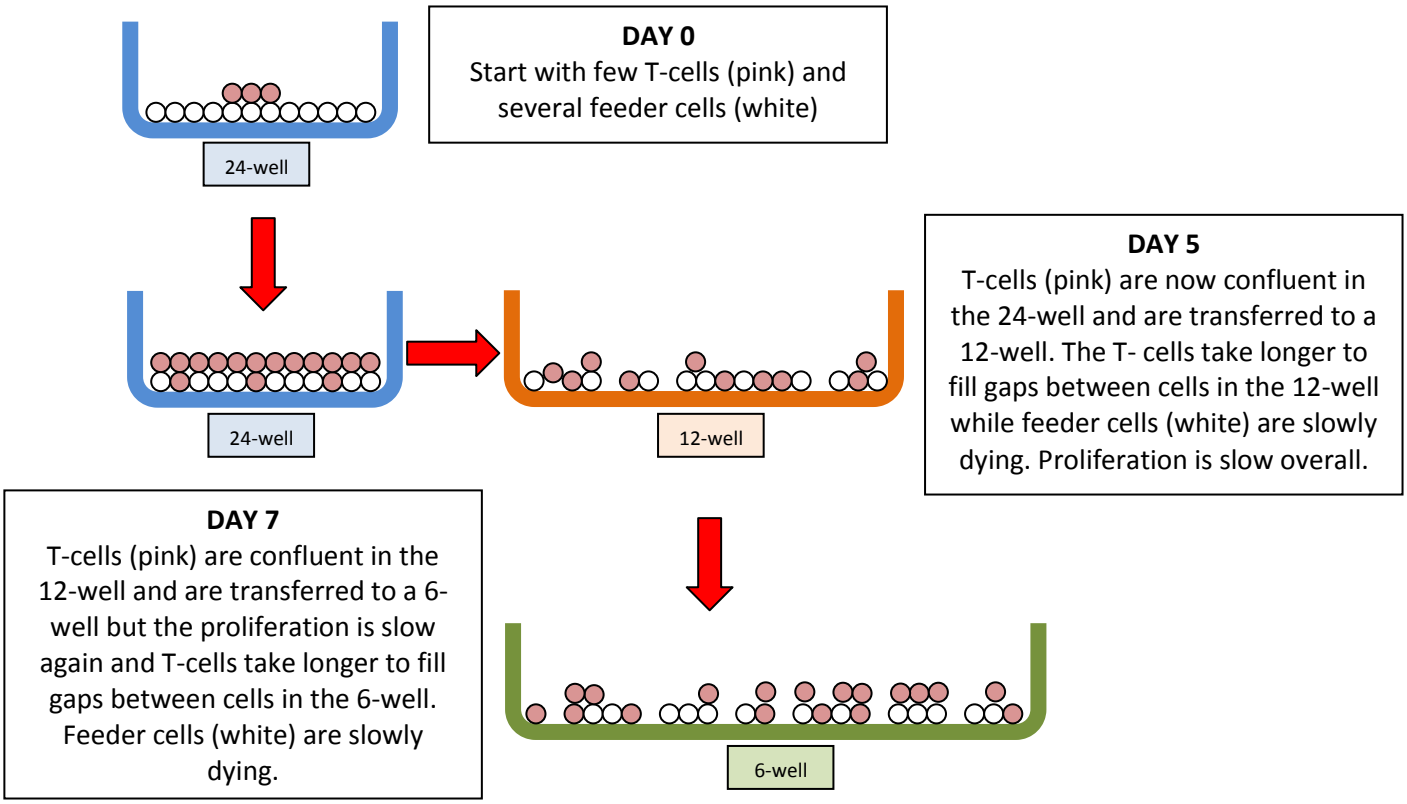
It appears that approach 2 and control have significantly better growth compared to the other approaches (1 and 3). The only difference between approach 2 and approaches 1 or 3 is that approach 2 consists of more 24-wells during the initial stages and is similar to the control during this period (figure 8.4, page 112). The difference between the control and approach 2 is the 6 well in approach 2.

What we can derive from this are the following points:

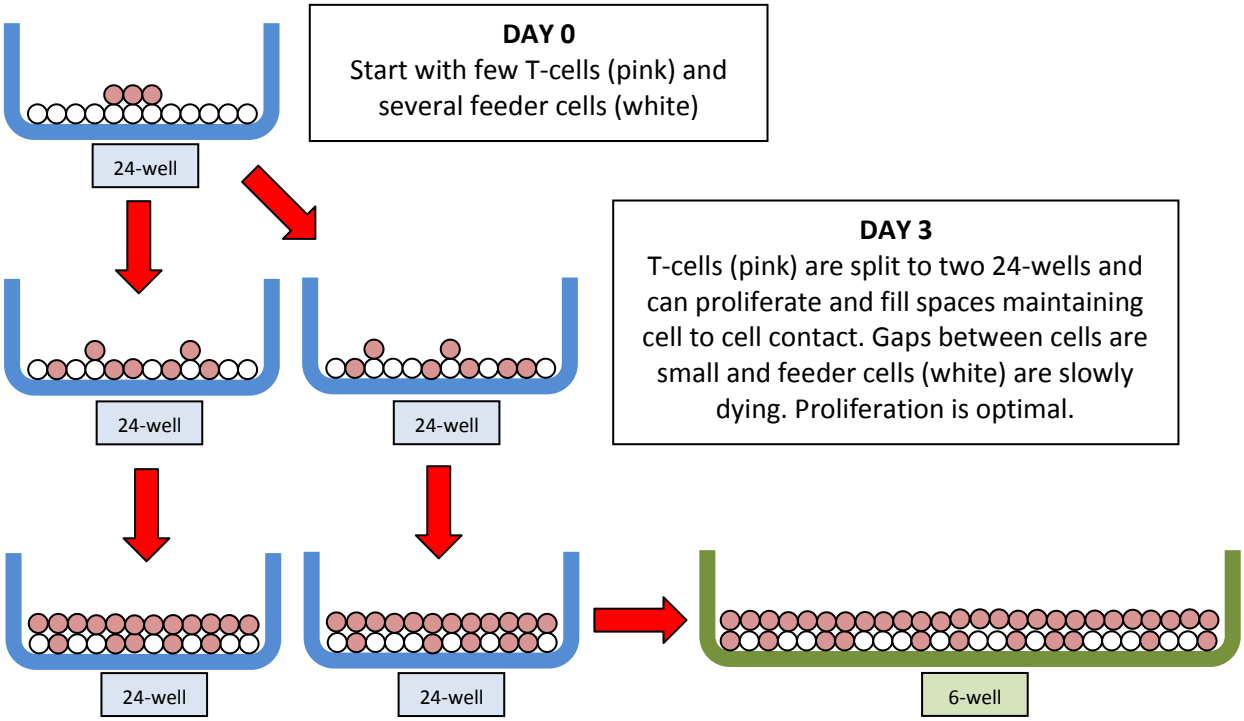
1. Changes in geometry do not affect T-cell growth during initial stages as long as the chamber is small (control and approach 2 are different initially but have similar fold expansion).
2. Smaller geometries are better during the initial stages of culture (at least for the first 6 to 7 days of T-cell culture as seen in control and approach 2).
3. Once T-cells start expanding and are in the proliferative phase they need lots of space and nutrients for growth hence larger geometries are needed at later stages of culture (6 well in approach 2).
4. The 12 wells (approaches 1 and 3) might be limiting growth as there might be reduced cell-to-cell interaction and larger spaces between cells (we don't know the rate of cell division at this stage and it may vary from donor to donor).

The flowchart below is a theory on why poor growth is seen in approaches having 12-wells during the initial 6 or 7 days of culture. The text boxes indicate conditions of the cells during each stage:

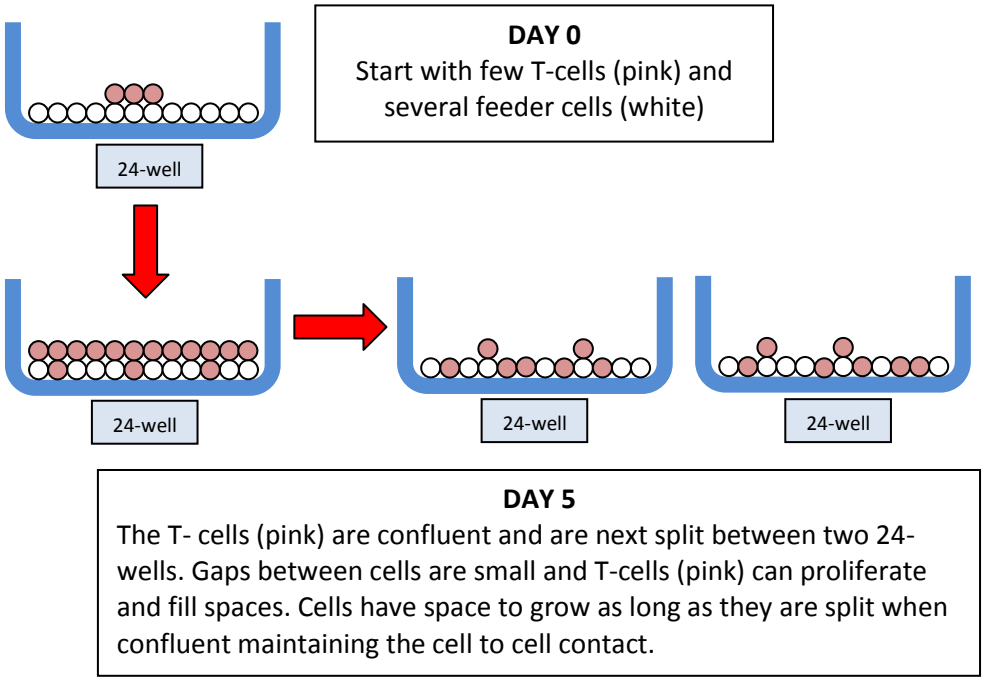
Approach 1 or 3:



Approach 2:



Control (24-well):



Flowchart 6.1 (previous page): Poor growth seen in approaches having 12 wells in first 6 to 7 days of culture. *T Lymphocytes growing in 24-well plates are exposed to surface areas that are easy to cover. This is the case with approach 2 and control where 24-wells provide adequate space for cell growth and is small enough to allow sufficient cell to cell interaction during the initial stage of culture (first 6 or 7 days). However, in approaches 1 and 3, the 12 well is too large and as a result reduced cell to cell interaction occurs which also impacts cell growth. This difference is magnified since cell growth is exponential and is seen at the end of culture on day 18 with significantly different fold expansions.*

In 24-well plate culture cell growth is not physically limited. This is because of the constant splitting of cultures done when necessary (depending on medium colour, confluency and size of cell clumps) hence, there is very less chance of cells running out of space or nutrients for growth.

It is however, difficult to judge when to split T-cell cultures in 6-wells as the cells tend to aggregate in the centre of the well instead of spreading out evenly as in a 24-well plate culture.

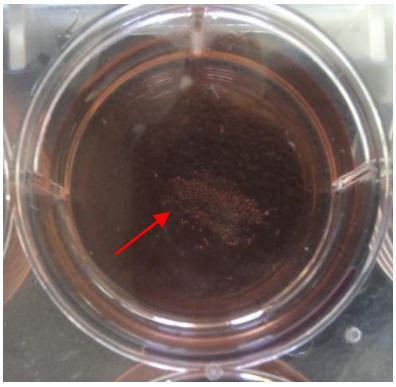


Figure 6.5: T-cells clumping in the centre of a well in a 6-well plate instead of spreading out (naked eye).

The medium colour (pH) in 6-well plate cultures change very slowly compared to 24-well plate cultures. These proved to be limiting factors in this experiment and to overcome this, the 6-well cultures were fed fresh medium once every 3 days irrespective of medium colour. The cell clumps were broken whenever possible to encourage spreading of cells and also restore cell to cell interaction. This was useful in promoting confluence in cultures growing in 6-well plates and also made it easier to determine when to split the culture into new wells.

Furthermore, the cell growth in these experiments correlate with findings in figure 5.10 (page 28) where the larger geometry in 24-well plates seem to bring about better growth over prolonged culture (18 days).

The fold expansion could also be correlated with the surface area used up by cells growing in the various approaches (figure 5.17, page 36). The surface area was determined by counting the number of confluent wells on day 18. The dimensions for each well in 24, 12 and 6-well plates were obtained from the manufacturer. All wells were confluent on day 18 making it easier to calculate.

There appears to be almost the same amount of surface area occupied by the control and approach 2 while the other two approaches (1 and 3) occupied significantly less area. This might suggest that cells are not growing at the optimum rate when they have a 12-well stage in the middle (like in approaches 1 and 3). A 6-well stage directly after the 24-well stage might be useful in avoiding physical limitations to the cell growth rate and also providing a lot of medium, nutrients and cytokines during the proliferative phase of the cells.

The functionality of the cells after culture in the four approaches is not affected and the cytokine secretion and CD107a expression is similar with no significant differences (figures 5.18 and 5.19, pages 36 and 37 respectively). This also holds true for the maturation states of the cells after the four approaches which are similar and consisting of mostly effector memory cells. The formation of immunological synapses is important for maturation of T-cells [7, 46, 47]. Hence the space between cells can affect this process which is possible in larger geometries. This was not the case in our experiments and can be attributed to the high cell densities developing due to proliferation of cells in all geometries.

It would be interesting if in-depth studies can be carried out to investigate the effect of geometry on: cytokine concentrations, accumulated metabolites such as lactic acid and concentrations of nutrients such as glucose over an entire 18 day culture period. Indeed factors such as diurnal variation and peak levels of these factors will have to be determined beforehand so that monitoring can take place at the right time points. These studies might provide further insight into the influence of geometry of the culture chamber on cell growth and the best approaches to expand T-cells.

Rough surfaces are better for T-cell growth and R30 has the best fold expansion overall

In earlier studies with material testing experiments (pages 20, 22 and 24) rough surfaces were found to influence T-cell growth. There was also a pattern where roughness R30 had the same growth as a 24-well plate culture on day 8 but after 18 days the growth was higher in 24-well plate cultures (page 28). We decided to further investigate this pattern of T-cell growth on the three roughness grades with a new set of blood donors. Tecoflex (TFX) was also included in the experiments to investigate if rough surfaces can overcome the negative influences of the material on T-cell growth.

In this experiment nine donors were used and T-cells were generated with the Pan T-cell stimulation protocol. The fold expansion (figure 5.22, page 40) again shows that rough surfaces in general are better for culturing T-cells compared to smooth surfaces. This is probably because of a

higher frequency of cell-to-cell interaction and better cytokine diffusion in cells growing on rough surfaces compared to smooth ones. What is interesting here is that there appears to be an optimum grade of roughness (R30) that has a significantly higher fold expansion compared to smooth surfaces (figure 5.22). The expansion on R30 is also higher than R45 growth (24.9 fold vs. 21.3 fold) proving that a certain grade of roughness is necessary to get the best growth. This pattern of growth was constantly observed in all our 8 day culture experiments where R30 would be the highest followed by R45 while R00 would have the lowest fold expansion.

When this experiment was repeated with EBV specific T-cells (using the EBV peptide stimulation protocol and culture for 18 days) the pattern of growth was slightly different. PS R30 still showed the highest fold expansion (figure 5.23, page 41). The roughness R45 maintained a fold expansion lower than R30 while the smooth surface (R00) had an expansion comparable to R45 on day 18. This is different to what we saw earlier with the experiment involving growth for 8 days (where rough surfaces (R30 and R45) showed better growth than smooth surfaces (R00)). While there might be a high frequency of cell-to-cell interaction among cells growing on rough surfaces, this advantage is also available to cells on smooth surfaces after some time when the cells have reached a higher mass during longer culture periods. This might be the reason growth on R00 was able to catch up with R45 growth.

Additionally this pattern was also visible on TFX too where TFX R30 had better growth than the other two surfaces (TFX R00 and TFX R45). This implies that roughness can overcome (to a small extent) the limiting influence of the TFX material on T-cell growth. Furthermore, this is also seen only after prolonged culture for 18 days.

T lymphocyte functionality does not seem to be influenced by the roughness which correlates with the findings from figures 5.11, 5.12 and 5.13. However, the lower cytokine and CD107a expression in T-cells grown on TFX indicate that material can influence T-cell functionality irrespective of the surface roughness (figures 5.24 and 5.25).

In a separate experiment EBV specific T-cells grown on polystyrene with three grades of roughness (R00. R30 and R45) show that there are significantly more multi-producers among CD4 and CD8 cells when grown on rough surfaces (figure 5.26). Multi-producers in our investigation are cells that are capable of secreting IFNγ and TNFα and expressing CD107a all at the same time when stimulated with EBV peptides. T-cells that are capable of producing more than one cytokine are known to be better at fighting infections compared to single producers [48]. This is another advantage rough surfaces have over smooth surfaces when culturing T lymphocytes.

T-cells require “continuous” surfaces for growth

All our previous studies involving rough surfaces and materials gave us a good idea of how T-cells interact and grow on surfaces. We decided to alter the surface by using electrospun fibres to grow T-cells. These are micro-structured surfaces constructed from fibres that are produced by a method called “electrospinning”. Any of the biocompatible materials in our previous experiments can be used to generate these fibres and hence provide a unique surface like seen in figure 6.6. Two types of alignment (parallel and random) are possible among the fibres. The number of layers and density of fibres can be modulated as desired but in our experiments these two variables were kept constant.

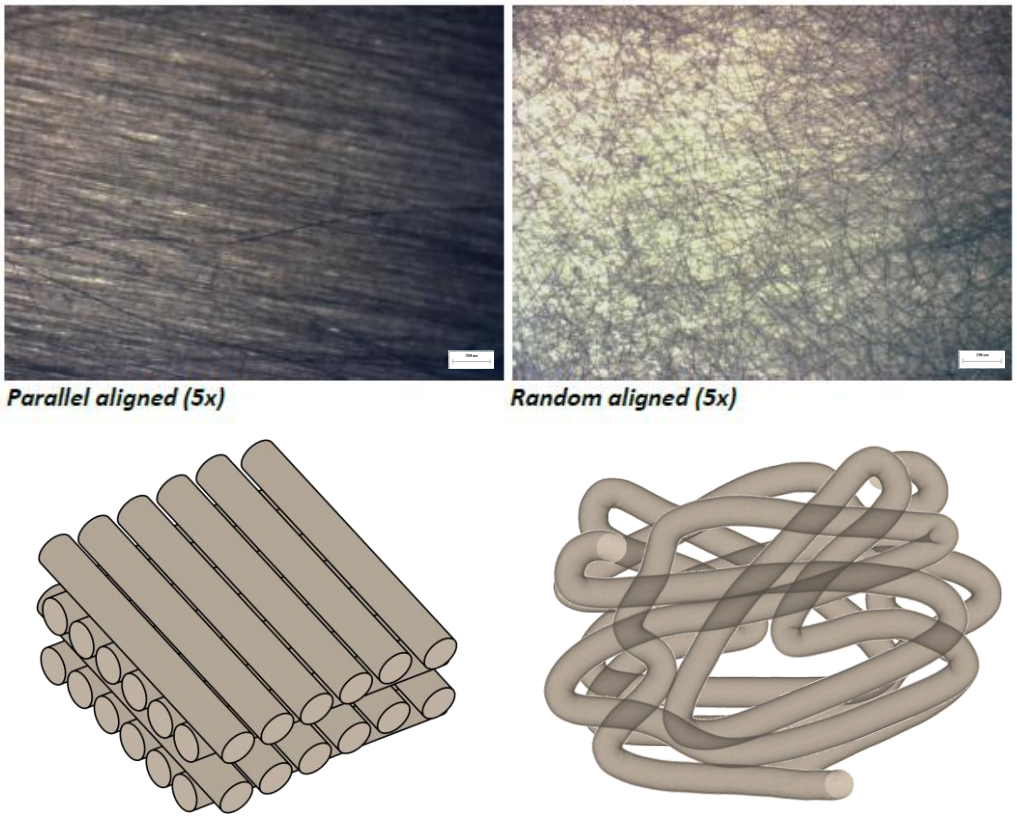


Figure 6.6: Electrospun material as seen through microscope and schematic representation.
These were placed at the bottom of material testing cups to provide a unique “micro-structured” surface for T-cell culture. The scale is 200µm in the two pictures.

These materials present a new type of “micro-structured surface” which is different from what the cells were exposed to in the previous experiments. The electrospun material is not a “continuous” surface. The area of contact with cells is different from a regular surface which has a relatively larger area of contact compared to electrospun surfaces (figure 6.7).

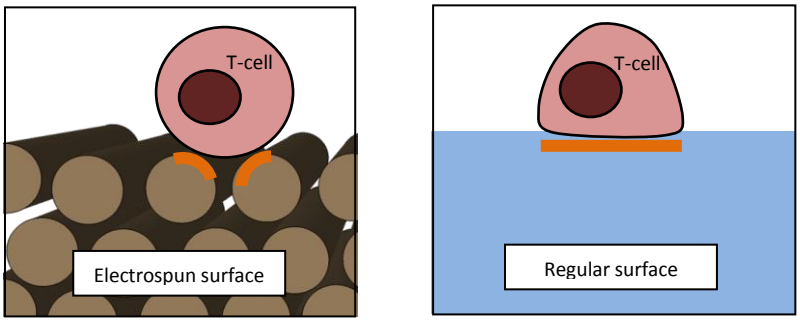


Figure 6.7: Continuous and non-continuous surfaces schematic. *(Orange line indicates area of contact) These images are assumptions based on findings. In-order to properly visualize T-cells on electrospun material and flat surfaces, SEM images would be appropriate. However, it is difficult to fix T-cells for SEM without disturbing their placement on a surface due to their semi-adherent nature.*

In our experiments, T-cells were generated using the EBV peptide stimulation protocol and culture carried out for 18 days. The resulting growth was very poor on electrospun surfaces irrespective of the fibre alignment (figure 5.29) compared to the PS R00 and 24 well plate culture which were made of the same material. It doesn't seem that material is negatively influencing cell growth since the proportions of CD4 and CD8 cells is maintained on both smooth and electrospun surfaces (figure 5.30).

The cell growth on day 5 (before the first splitting of culture) appears to be normal with clump formation on parallel aligned electrospun fibres which is similar to clumps on smooth surfaces (figure 5.31). It wasn't easy to microscope these materials because of difference in focal depth between individual fibres making it hard to determine clump formation on random aligned fibres (figure 5.31). After day 5 it was impossible to visualize clump formation on both random and parallel aligned fibres due to blockage of light from lower fibre layers.

When manufacturing these surfaces the gaps between fibres were made very small (2 to 5 µm) approximately half the diameter of a fibre. They were also adjusted so that cells would not fall through them by arranging fibres in layers that are aligned at 90° to each other as seen in parallel aligned fibres (figure 6.6). However T-cells are known to migrate between gaps by squeezing through as they do during inflammation induced trans-endothelial migration [49]. They are also known to migrate through pores which are 3 to 5 µm in size [50]. This is extremely small considering the size of an average lymphocyte which is around 10µm. However, it is very possible that T-cells are forced to migrate into the gaps since we use IL-2 in our medium which mimics inflammatory conditions. The result would be that once cells squeeze through the tiny gaps they die due to damage of cellular structures and loss of cell-to-cell interaction (figure 6.8).

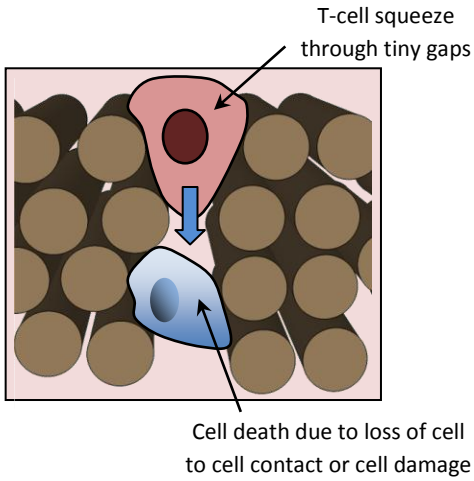


Figure 6.8: Probable causes of cell death in T-cells growing on electrospun fibres.

The feeder cells we use in our cultures could also get trapped in-between fibres in a similar manner and die earlier than usual reducing the amount of cell-to-cell interaction normally available during the initial stages of culture.

After harvesting cells on day 18, the fibres were stained with Tryptan Blue and viewed under the microscope. No live or dead cells could be visualized between the fibres (image not shown). It is possible that most of the cells that manage to move between fibres would have disintegrated leaving behind only cell debris that gets washed away during medium changes. We did not further investigate the cause of cell death on electrospun materials since it presents technical issues that need to be addressed first (culture conditions and fibre manufacture) and at the moment it does not seem feasible to use them to culture T-cells.

In order to properly visualize the orientation of cells on these fibres a scanning electron microscope (SEM) may be used in future studies. However, currently it is not possible to prepare T-cells for SEM due to their semi-adherent nature. The slightest turbulence in the medium causes the cells to move about and become dislodged from the surface. This might also dislodge cells that are in the process of squeezing through the gaps.

Repeating these experiments with single layered electrospun fibres placed on solid surfaces might be a solution since there are no gaps that can cause cell death but this would be similar to our roughness testing experiments where we use solid surfaces that are physically modified to present different grades of roughness. Technical problems arise if we try to reduce the gaps between fibres even further and would require more sophisticated machines to generate such structures.

Section 3:

T-cell and observer interaction

T-cell generation and growth

T cells can be expanded using a fixed splitting schedule

The culture of T lymphocytes requires a great deal of observation and interpretation of culture conditions from visual cues. These consist of the following:

1. Confluency of T lymphocyte culture
2. Medium colour (orange, yellow or pink)
3. Size of cell clumps
4. Density and colour of clumps (dark or light)

A combination of the above is used by the observer to judge the time point for splitting of culture into fresh wells (figure 6.9). Hence the observer can influence proliferation by the way T cells are manipulated during culture. Thus human error and observer bias is unavoidable and do occur occasionally when expanding cells in a laboratory environment. T lymphocyte proliferation is also dependant on culture conditions and health of the original blood donor. All of these in combination could influence proliferation rate and overall fold expansion.

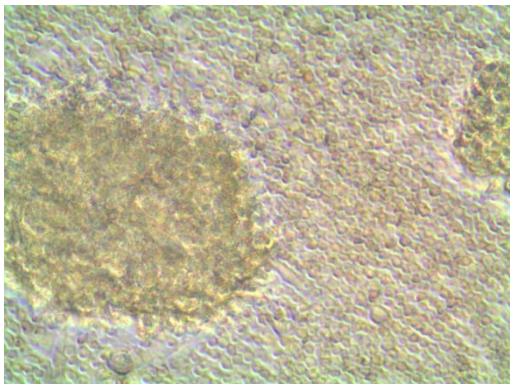


Figure 6.9: Confluent T Lymphocyte culture growing on a 24-well plate.
Large clump can be seen on left side and almost no space is available between cells. This would be an ideal time to split half of these T cells into a fresh well so that they have more space to proliferate.

There is usually a high amount of variation between the overall fold expansions from donor to donor making it harder to predict the final cell number that can be attained (figure 5.32). We decided to expand T-cells according to a schedule where splitting of culture would take place at fixed time points (please see “fixed splitting schedule” in “methods”, page 117) minimizing the need to judge culture conditions. A schedule for splitting may eliminate observer bias and in turn provide a uniform method for T cell culture expansion across cell lines from different donors. When EBV

specific T-cells were exposed to the fixed splitting schedule the amount of variation between donors reduced (figure 5.33).

We also compared both these methods by generating EBV specific T-cells using the EBV peptide stimulation protocol and exposing cells from donors to both methods of expansion (fixed and normal). The result was that cells expanded using the fixed splitting schedule showed less variation in cell number and fold expansion compared to those expanded the normal way (figure 5.34). It is possible to estimate the final cell number and fold expansion ranges using this method of expansion (fixed splitting).

However, cells expanded the normal way show higher overall cell numbers compared to cells expanded by the fixed splitting approach. Thus the schedule needs to be further optimized to reach the high cell numbers which is currently only possible using the normal expansion method. As future studies perhaps other cell types (eg: B cells and T Reg cells) can be exposed to the fixed splitting schedule to observe if it is possible to standardize the expansion process.

There are perhaps a few limitations to expanding cells using the fixed splitting schedule which may not be easily overcome:

- T-cells capable of higher proliferation rates may not grow at those rates if cultures are not split when confluent. This is possible in the fixed splitting schedule where cultures are split at fixed time points rather than when they are confluent. This may cause cells to accumulate leaving less space for overall cell proliferation. It may however; increase cell to cell interaction available to other slow growing cells.
- Cells with lower proliferation rates risk a decrease in cell to cell interaction when cultures are split before they are confluent. This is due to the increased space available after splitting which may also lead to higher cell death than normal.
- In order to be effective the fixed splitting schedule requires T-cells to proliferate at similar rates from donor to donor or at least within a small range of proliferation rates. This may be difficult to assess since several factors can influence T-cell growth (e.g. donor age, health status, etc). However as seen from the results, it may be possible to obtain a uniform growth rate across most donors with less variation in the final product in terms of cell number or fold expansion.

The experiment was repeated again but this time using rough surfaces to expand T-cells using both methods of expansion (fixed and normal). Again the cells expanded using fixed splitting

schedule showed less variation in fold expansion compared to those expanded the normal way (figure 5.35). However, interestingly the rough surface R30 no longer has the best growth when expanded using the fixed splitting schedule. Usually the growth on R30 was higher than R00 and R45 when cells were expanded using the normal method (figures 5.22 and 5.23). The T-cells grown in 24-well plates show lower fold expansion when expanded with the fixed splitting schedule compared to those expanded by the normal method (figure 5.35).

There are several factors influencing the cells growing on rough surfaces and is a good example of multi-factorial influence on T-cell growth which was also seen in the experiment comparing two bioreactors (page 94). It is not clear why in this case R30 has poor growth when expanded using the fixed splitting schedule. Perhaps we need to first optimize the fixed splitting schedule to generate higher cell numbers (similar to the normal method of expansion) before we can understand cell growth on R30 using the fixed splitting schedule.

Peptide pools

Antigen specific T lymphocytes are generated using the EBV peptide stimulation protocol. During the stimulation step, pre-processed peptides are loaded on the cell surface of Antigen Presenting Cells (APCs) (dendritic cells, macrophages and B cells). These in turn are capable of stimulating CD4 and CD8 cells that are specific to the peptide presented [51]. In our case we use peptides derived from the EBV virus and according to the manufacturer instructions 1μg/ml is used for stimulation.

We decided to titrate down the concentration of peptide needed to stimulate T-cells and hence lower the cost of T-cell generation. Three concentrations were investigated 1μg/ml, 0.5μg/ml and 0.1μg/ml. We found that it was possible to stimulate T-cells and grow them for 18 days using the lowest concentration of peptide (0.1μg/ml) (figure 5.36). The proportions of CD4 and CD8 are also unaffected after 18 days culture after lowering concentration of peptide used for stimulation (figure 5.37). This is important since as mentioned earlier the proportions of CD4 and CD8 cells need to be maintained to ensure a proper immune response. Surprisingly the functionality of T-cells is not affected by lower concentrations of peptide as evident by the IFNγ secretion (figure 5.38), TNFα secretion (figure 5.39) and CD107a expression (figure 5.40). These results taken together mean that we can safely use the lowest concentration of peptide (0.1μg/ml) to stimulate and culture EBV specific T-cells and in the process also lower the cost of T-cell generation. The cost becomes important when large scale generation of T-cells is carried out such as in a GMP facility.

Section 4:

T-cell manipulation during inoculation, culture and harvesting

T-cell manipulation during inoculation, culture and harvesting

T Lymphocytes require constant manipulation during the main stages: Inoculation, culture and harvesting. During in-vitro culture, T lymphocytes tend to form clumps and often aggregate in niches and corners (as discussed earlier in the roughness experiments). This tends to prevent cells that are trapped in clumps from being exposed to nutrients and growth factors that are available in the medium. Also gas diffusion (that is required for metabolism) might not be available to cells that are trapped within clumps. Hence, there is a need for constant manipulation to break clumps and homogenize the medium during the three main stages of inoculation, culture and harvesting.

Current techniques for T-cell manipulation

The manipulation is usually done manually under a sterile bench with pipettes by the investigator. This works well for open systems such as the 24-well plate culture, but might not be feasible for a closed system such as a bioreactor. In such a system the manipulation will have to be done without opening the culture chambers in order to maintain sterility of the contents. Additionally, cells must not be left behind during harvesting since T-cells tend to adhere to the bottom of the culture chamber. The manipulation will have to be gentle so that cells are not damaged by the action of the manipulation process.

The need for manipulation within bioreactors has been accomplished previously by using stirrers which enabled large scale manufacture of antigen specific T-cells [52]. However studies also show that T cells downregulate the IL-2 receptor when exposed to stirrers resulting in a slightly lower fold expansion compared to T-cells grown in static cultures [8]. This downregulation of IL-2 receptor is aggravated when higher speeds are used. They also show that the presence of bubbles in the culture can reduce the fold expansion even further [8]. Additionally, since IL-2 receptors are important in the immunological response this might have an influence on T-cell cytokine secretion but the mentioned studies do not comment on this aspect. However, the T-cells retained their cytolytic capacity even after exposure to stirred bioreactors [52].

Then studies have also shown that turbulent flow in the medium can activate γ/δ T lymphocytes compared to laminar flow [9]. This is undesirable when culturing antigen specific T-cells since over activation might also hamper the functionality of T-cells. These findings together indicate a stirrer is not favourable for our culture system since there are more disadvantages than advantages associated with their use.

Then keeping T-cells constantly suspended, such as in a rotating wall vessel (RWV) bioreactor was also not feasible as studies have previously shown that most T-cells die within the culture after more than 8 hours exposure to such kinds of bioreactors and functionality including IL-2 secretion was also noticeably affected [10, 53].

Perfusion bioreactors have been successfully used to grow T lymphocytes where cells are exposed to constant wave motion with no significant affects on their cytokine secretion or cytolytic abilitiy [54-56]. However large numbers of T lymphocytes (approx. 10^6 cells/ml) are required before culture can be started in these systems and since our initial cell numbers are very low (100,000 to 500,000 cells), it is not feasible to use this kind of system either. Hence, we decided on a compromise accounting for all these factors and choose to use a mixing-table that incorporates most of the features we needed.

Mixing table for breaking cell clumps and homogenizing culture contents

The mixing-table is a simple set-up and if required could be easily integrated into several existing bioreactor systems. The device Mixmate® from Eppendorf was used which had an adapter that can be used to hold a small culture vessel on top of the device. We planned to use the device to break cell clumps and homogenize culture contents during inoculation, culture and harvesting stages without having to open the culture system.

We tested the mixing-table with antigen specific T-cells and compared it to the manual method using pipettes to break cell clumps and homogenize the medium. Depending on the speed and running time of the machine, all of the cell clumps could be broken easily with no effort from the observer (tables 5.2, 5.3, and 5.4, pages 58 and 59). Larger culture vessels such as the 6-well plate could do this at slower speeds since the circular motion of the machine caused the medium to develop large waves and dislodge cell clumps (Table 5.4). A higher speed resulted in splashing in larger vessels but was not a problem in smaller vessels such as the 24-well plate where higher speeds were required to break cell clumps in shorter time periods (Table 5.2).

The device has proved to be an ideal solution to manipulating T-cells in a closed system. During T-cell culture the mixing-table can be adapted to the fixed splitting schedule and used at fixed time points instead of being used constantly throughout the culture period. This limits the influence on cell growth and can avoid most of the complications mentioned previously with other systems. This can also be used across cell lines from different donors so that end-results are more reproducible and uniform.

Section 5:

Comparison of existing systems

Comparison of existing systems

We analyzed the growth of T-cells in two bioreactors in order to better understand the limitations associated with T-cell growth in different culture systems. The limitations of each system are unique and hence present culture conditions which are slightly different to each other. However, these systems cannot be compared to our 24-well plate cultures which we used as a standard in our experiments for comparison. This was also useful to identify differences in factors influencing cell growth in each system.

The two bioreactors we selected for our experiments demonstrated features that are desirable when culturing T-cells and feasible for GMP use if they prove successful. The first bioreactor "G-Rex" was from the Wilson Wolf Manufacturing Corporation, USA and has been specially designed to culture antigen specific T-cells. The bioreactor consists of a single chamber with a gas permeable membrane floor made of modified Silicon (figure 6.10). The T-cells resting on this membrane have a direct supply of gases which allow an increase in medium height without affecting gas exchange to the cells. Studies show that the G-Rex bioreactor is capable of a 20-fold higher output than the 24-well plate cultures which was attributed to lower cell death rates in the G-Rex bioreactor compared to the 24-well plate cultures [57].

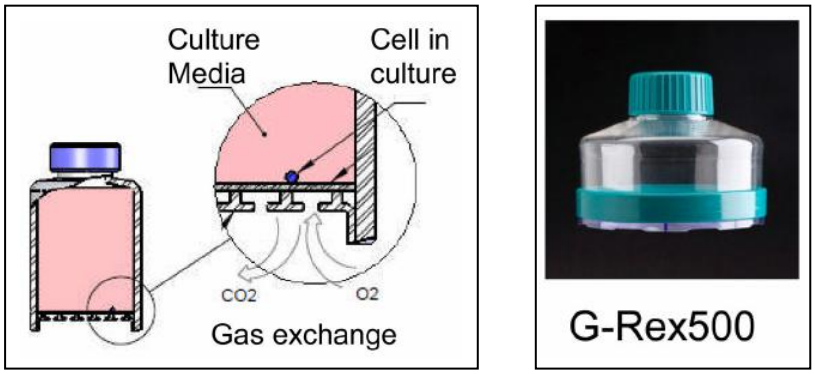


Figure 6.10: G-Rex bioreactor and gas-permeable membrane floor.
Adapted from paper: Accelerated production of antigen-specific T-cells for pre-clinical and clinical applications using Gas-permeable Rapid Expansion cultureware (G-Rex). J Immunother. 2010 April, Rooney et al.

The second bioreactor in our experiment was a perfusion bioreactor (hollow-fibre membrane bioreactor) that was previously used to culture primary human hepatocytes [58]. The device was connected to a perfusion system that was electronically controlled and used capillaries to deliver gases (air and CO_2) and medium to the culture chambers (figures 8.10 and 6.11). Since this is similar to the G-Rex system in terms of gas exchange, we decided to compare the two bioreactors with the

standard 24-well plate cultures. Additionally, the cells in the 24-well plates were expanded using both the fixed splitting schedule and normal method for further comparison.

G-Rex bioreactor was the best system overall

It appears that the best system out of the four (G-Rex, perfusion bioreactor, 24-well (normal) and 24-well (fixed)) was the G-Rex bioreactor. The fold expansion and CD4 / CD8 proportions was found to be the most optimal in the G-Rex while the worst system was the perfusion bioreactor which had very poor growth despite a good proportion of CD4 and CD8 cells.

Perfusion Bioreactor

It is unclear what could be the reason for the very poor growth in the perfusion system and it is more likely that a combination of several factors is affecting cell growth in this bioreactor. Firstly, the gases supplied in the perfusion bioreactor are a mixture of air and 5% CO_2 which is the same as the G-Rex bioreactor which obtains its gas supply directly from the incubator atmosphere. The oxygen tension in a regular incubator is at 20% of air saturation, while studies have shown that oxygen tensions greater than 50% tend to hamper T-cell growth [4]. The same oxygen tension of 20% was also found to have the best proliferation rates for primary lymphocytes compared to physiologic oxygen levels at 5% [6]. However, this level at 20% was found to have an influence on intracellular NO (iNO) levels and CD69 expression which might not be favourable for T-cells during extended culture [59]. The growth of T-cells should be equally affected in the G-Rex and 24-well plate cultures as both are exposed to the same atmosphere as the perfusion bioreactor which does not explain the poor growth in the latter.

The constant perfusion of medium through the capillaries in the perfusion bioreactor might be another factor that influences growth since the quantity of cytokine and growth factors secreted by T-cells is very less especially at the start of culture. The amount of cytokine and growth factors needed becomes greater when the perfusion system is switched on. This is because the cytokines get washed away by the perfusion system which affects cell growth. During culture, the medium (as in all four systems) was supplemented with 100 IU of IL-2. A low speed of continuous perfusion at 3ml/min was set so that cytokines are recirculated back to the cells in a closed circuit. Fresh medium was added to this circuit only when samples were taken out of the bioreactor. However, it may be possible that cytokines secreted by the cells are adsorbed on tubing and other surfaces inside the

bioreactor. Fresh medium supplemented with foetal calf serum and IL-2 was perfused through the perfusion bioreactor for 2 days before cells were inoculated into the system. This was intended to calibrate the bioreactor to our culture conditions and also allow protein adsorption to take place and cover most or all of the culture surfaces inside the bioreactor.

Since the amount of cytokine secreted by T-cells is very low during the initial stages of culture, it is difficult to determine if they are really being adsorbed onto the tubing walls inside the perfusion bioreactor. Measurement of IL-2 levels by cytometric bead array in samples taken from the bioreactor throughout the culture period show that IL-2 levels were very low compared to 24-well plate cultures (data not shown) hence this might be responsible for the poor growth observed. Unfortunately this is un-avoidable in the perfustion bioreactor due to its construction. This will be a limiting factor that is difficult to overcome unless we artificially boost cytokine levels inside the culture chamber, for example, by using biomaterials for a controlled release of cytokine to maintain T-cell populations [60].

The floor surface area of the G-Rex system is much greater (10 cm^2) compared to the perfusion bioreactor (3.1 cm^2) which limits the amount of "flat" space available for cells to grow in the perfusion bioreactor. However, T-cells are known to form clumps and tend to aggregate as long as there is sufficient gas supply, hence the surface area alone might not be the cause for the low fold expansion. Additionally, the capillary network (figure 6.11) that traverses the culture chamber provides additional surfaces on which cells can attach and grow which increases the surface area in a smaller space compared to a single flat surface of the same size.

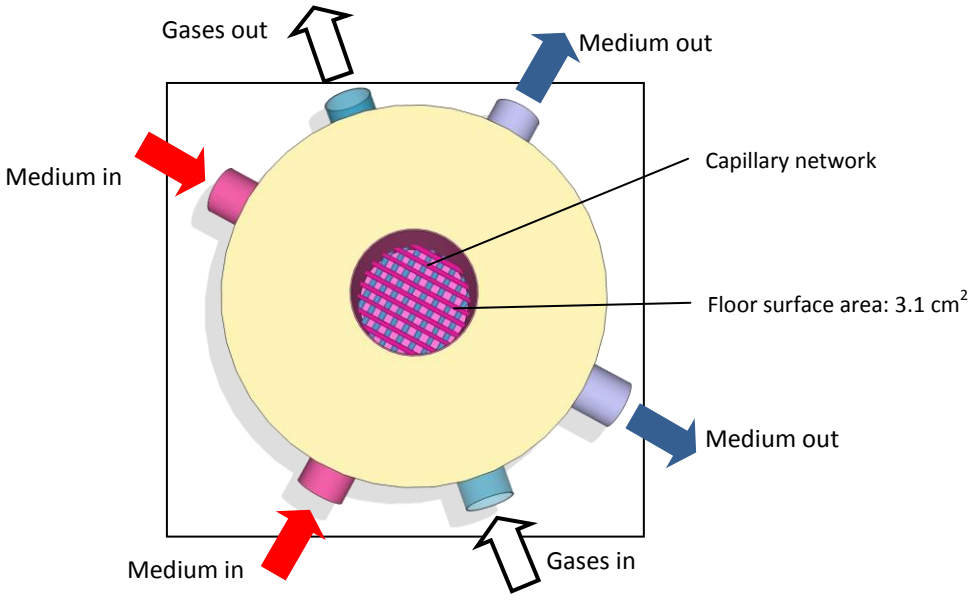


Figure 6.11: Capillary bed in perfusion bioreactor provides further surface area for cells to grow.

G-Rex bioreactor

Apparently what works in favour for the G-Rex system is the gas permeable membrane that allows for larger volumes of medium to be used (30 ml) without affecting gas exchange in T-cells (figure 6.10) which means more nutrients and cytokines are available for growth compared to a 24-well plate culture system [57]. Once we filled a 40ml G-Rex bioreactor with 30 ml of medium, there was no need for a medium change for the next 5 to 6 days. This is advantageous since the cells grow without much manipulation which in the long run limits physical damage to cells allowing them to multiply freely. Studies have shown that this bioreactor was able to outperform the 24-well culture system in terms of ease of use, cost of production and lower cell death rates in T-cells [57]. The findings in our experiments are to be expected since this bioreactor has been designed primarily for culturing antigen specific T-cells, keeping in mind the limitations associated with large scale culture of T-cells.

T-cells grown in the G-Rex bioreactor have the best functionality and CD4/CD8 proportions

The functionality of T-cells from the four systems was also quite different in terms of IFNγ and TNFα secretion. The T-cells grown in the perfusion bioreactor had the lowest levels of IFNγ and TNFα secretion compared to the G-Rex bioreactor and 24-well plate cultures. The expression of CD107a followed the same pattern where the lowest amounts were expressed by T-cells grown in the perfusion bioreactor compared to other systems. Together this meant the perfusion bioreactor was not suitable for culturing antigen specific T-cells for immunotherapeutic applications unless improvements are made in several areas, for instance, the cytokine levels inside the bioreactor and surface area available for cell culture. The best system overall was the G-Rex bioreactor which had a better fold expansion, CD4 and CD8 proportion and T-cell functionality compared to both of the 24-well plate cultures (fixed and normal expansion). This was all accomplished by growing T-cells on a gas permeable membrane in the G-Rex despite providing lesser surface area than a 24 well plate (10 cm^2 vs. 46 cm^2 respectively).

Summary of findings and Outlook

Summary *of overall findings* **and outlook**

The influence of several factors together can have a different effect on T-cell growth compared to each factor on its own. This was proved in the experiment discussed earlier (page 94) with the two bioreactors (G-Rex and perfusion bioreactor) where despite both systems having similar culture conditions the T lymphocytes were different in terms of both functionality and fold expansion. Hence the design of a bioreactor for culturing T-cells needs to be carefully planned out with the findings from this study keeping in mind that these factors together may affect T-cell growth differently depending on the culture system. This means we still need to investigate how these factors together play a role on T-cell growth and perhaps the building of a prototype culture system is important before we proceed any further.

Based on all of the findings from all the previous experiments, we can derive certain specifications that need to be adhered to when designing a culture system for T-cells. They are as summarised in figure 7.1.

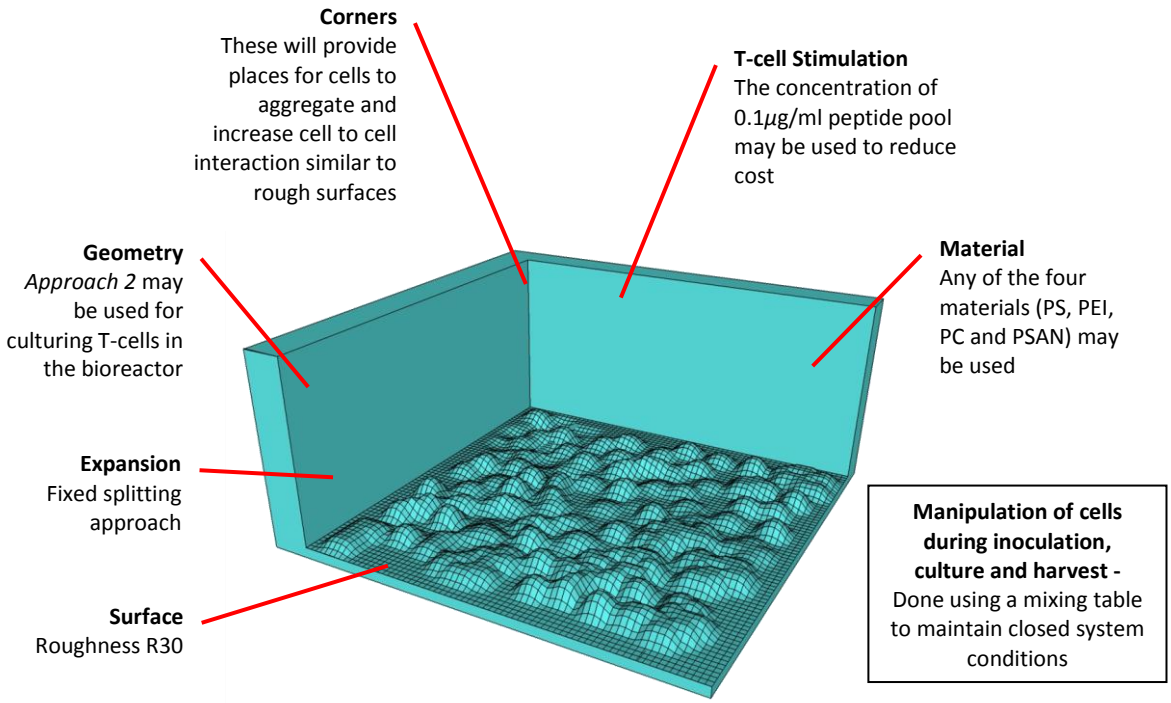


Figure 7.1: Summary of specifications for designing a bioreactor for T-cells.

When considering the material that needs to be used for building a culture system, the four (PS, PC, PEI and PSAN) we found to be compatible with T-cell growth may be used. Perhaps a combination of these materials might also be safely attempted. The culture surface for T-cells will definitely have to be a rough surface which has proved to be superior to a flat surface. However, rough surfaces might not have a good growth if combined with the fixed splitting schedule (as seen

with the experiment on page 51). This might be a problem with optimization of the fixed splitting schedule and needs to be further investigated before completely rejecting the idea. The bioreactor chambers will have to follow the approach 2 pattern of geometry to maintain cell to cell contact and also give T-cells ample space to grow from the earliest time point. During inoculation, culture and harvest, cell manipulation can be safely carried out using a mixing table such as the one used in page 118. While at the start when generating antigen specific T-cells, the lowest concentration of 0.1µg/ml peptide may be used without any loss in cell growth or function. This would also help in reducing the overall cost of generation.

In retrospect a wise choice would be to closely mimic the natural environment of T-cells where they are known to have the best proliferation namely, the lymph node and thymus. Earlier studies hoping to mimic this have been carried out with a human lymph node model (HuALN) [61, 62]. However, these systems were designed primarily to study the influence of drugs on cellular and humoral immune responses in terms of cytokine, antibody and cell activation. While in our case T-cell generation is the main aim along with a system to easily harvest T lymphocytes without cellular damage or cell loss, which is hard to do with an artificial lymph node. This limits the complexity of bioreactor that can be constructed for our purposes making systems with capillaries and gas tubing unfeasible for our purposes.

In future, studies about the interaction between T-cells and materials in terms of protein adsorption and oxygen plasma surface treatment may also be attempted. The study of “material chemistry” and its influence on T-cell growth and function might give us further clues as to why Tecolfex had such poor growth. The arrangement of T-cells on rough surfaces and electrospun surfaces must be studied (perhaps via SEM) which will help in understanding T-cell affinity for niches and corners during culture. Micro-structured surfaces (electrospun material) were found to negatively influence T-cell growth (page 45). It would be informative to study other types of surfaces which have regular microstructure patterns such as squares, rectangles, circles and triangles. This would make quantification of the influence of microstructure on T-cell growth much easier and reproducible. As an afterthought, materials that physically change during culture would be ideal for constructing our culture system as seen from the geometry testing experiments. Shape memory polymers seem to offer this feature and perhaps it would be good to add them to our line-up of materials in the material ↔ T-cell interaction experiments. It might be possible to have these materials decrease in roughness over time where at the start of T-cell culture we observe a roughness of R45 and this roughness decreases to R30 towards middle of culture so that T cells have lots of niches for optimum growth. The possibilities are endless and we still have a lot of work to do!

Materials and Methods

Materials and Methods

1. PBMC sources

In all experiments either **buffy coats** or **fresh blood** were used to supply PBMCs (Peripheral Blood Mononuclear Cells). The choice of source was based on the intended experiment however; there is no difference between the two sources since both are derived from fresh blood. The **buffy coats** were obtained from the Red Cross in Wannsee, Berlin, Germany while **fresh blood** was collected from healthy donors (20ml).

Buffy coats:

Once buffy coats were obtained they were drained into 250 ml tissue culture flasks and a portion of this was kept aside for CD3 depletion (10 ml – 20 ml depending on the experiment - please see "*CD3 depletion and freezing*"). The rest of the buffy coat was diluted with PBS (Phosphate Buffer Solution) in the ratio 1:1. Then procedure for Biocol separation of PBMCs was followed (see below).

Fresh blood:

Healthy donors were used and 20ml (per donor) was taken and exposed to CD3 enrichment cocktails (please see *"CD3 enrichment"* section). Then procedure for Biocol separation of PBMCs was followed (see below).

Biocol separation of PBMCs from buffy coats or fresh blood:

The diluted Buffy coats (or fresh blood after CD3 depletion) were carefully layered over Biocol (previously added to 25 ml falcon tubes). The proportions used were: Biocol 1/3 + diluted buffy coat or fresh blood 2/3. Depending on the volume of buffy coat or blood, several 25 ml falcon tubes were prepared.

The tubes were then gently placed in a centrifuge with the following settings: 24°C, spin at 1000 g for 20mins with centrifuge breaks off. After this the white Biocol layer is extracted with a pasture pipette and washed twice with PBS. The cells were counted and re-suspended in complete medium (1645 RPMI (Biochrome) with 10% FCS (foetal calf serum) and 5% Penstrep (Penicillin / Streptomycin (Biochrome)). If the PBMCs were isolated from **buffy coats**, they were allowed to rest for one day before stimulating with **EBV peptides** (please see T-cell generation using *"EBV peptide stimulation protocol*"). For **fresh blood** the PBMCs

were next stimulated with **MACS iBead particles** (please see T-cell generation using **"***Pan T-cell stimulation protocol"*).

2. Preparation of EBV peptides

Epstein-Barr virus (EBV) peptides were obtained from JPT Peptide Technologies, Berlin, Germany. These are overlapping peptides that are derived from proteins expressed in EBV-transformed lymphoblastoid cell lines (LCLs) and are target antigens for cytotoxic T-cells [11]. They consist of the Epstein-Barr nuclear antigens (EBNA 1, 2 and 3c) and latent membrane proteins (LMP1 and 2) both of which are expressed during the latent phase of the EBV infection. While the basic leucine zipper nuclear factor 1 (BLZF1) is expressed during the beginning of the lytic cycle and is targeted by EBV specific T-cells during active disease [12].

Table 8.1: Epstein-Barr virus peptide pools.

	Protein name	Number of peptides	NCBI entry	Concentration used
1	EBNA 1	158	YP_401677	1µg / ml
2	EBNA 2	119	CAD53395	1µg / ml
3	EBNA 3c	265	YP_001129465	1µg / ml
4	LMP 1	94	YP_401722	1µg / ml
5	LMP 2	122	YP_401631	1µg / ml
6	BLZF 1	59	YP_401673	1µg / ml

The peptide pools were prepared for use by dissolving the contents of each mixture in Dimethyl Sulfoxide (DMSO) and sterile water in proportions of 50% each to make a final concentration of 250 µg /ml. This mixture was stored at -80 °C. When stimulating cells the peptide pools were further diluted to 0.1 µg /ml using sterile water and then added to PBMCs. The cells were then incubated for 6 hours at 37 °C in complete medium before the next step.

3. Preparation of MACS iBead particles

The kit was obtained from Milteny Biotech. It contains special kind of particles which serve as human pan-T-cell stimulators. These particles have antibodies against CD3, CD28 and CD2 which are attached to the particle surface using biotin.

Preparation of MACS iBead particles was done as per manufacturer's instructions:

Briefly, 100 µl of each: Anti-CD2 biotin, Anti-CD3 biotin and Anti-CD28 biotin is mixed together and exposed to 500 µl iBead particles (100 x 10^6 iBead particles). Next 200 µl of PBS with 0.5% FCS is added to make the mixture up to 1 ml. This is then incubated for 2 hours at

2-8 °C under constant gentle rotation using a MACSmix tube rotator (4 rpm – slowest). After this the mixture may be used within 4 months and stored at 2-8 °C.

4. **T-cell generation:**

Two types of protocols were used for T-cell generation depending on the source of PBMCs:

a. *Buffy coat sources*: **EBV peptide stimulation protocol** (see below)

b. *Fresh blood sources*: **Pan T-cell stimulation protocol** (see page 107)

The protocols are different from each other and have only one common step: "Biocol separation of PBMCs from buffy coats or fresh blood" (please see page 102). These two generation methods were just for comparison and no significant difference was actually seen in T cell growth between the two methods during our experiments.

a. EBV peptide stimulation protocol *(for buffy coat sources)*:

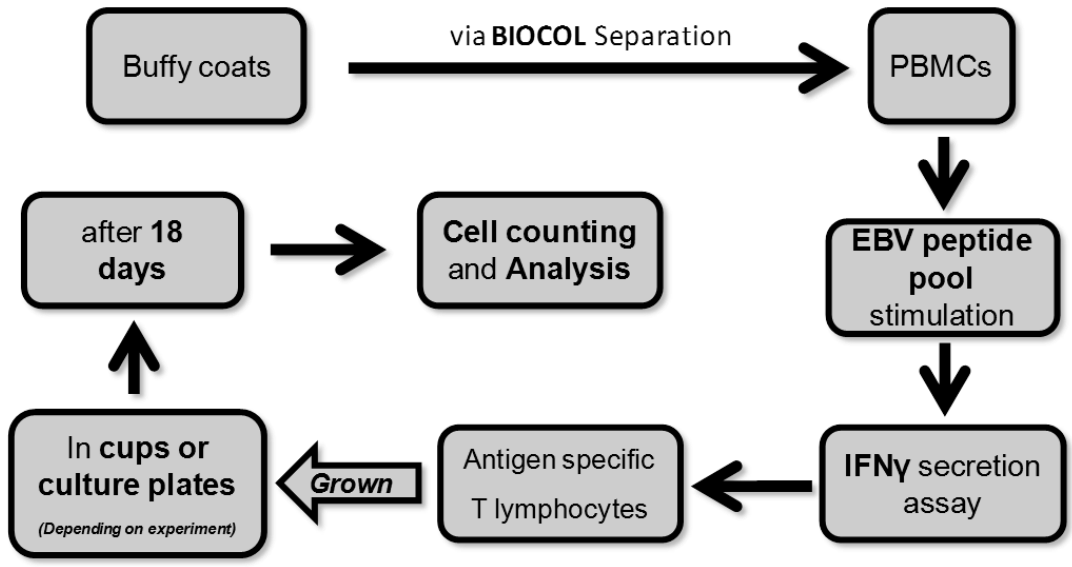


Figure 8.1b: EBV peptide stimulation protocol.
*Description in brief (Please see other sections in "methods" for detailed explanations of each step in **bold**):*
Buffy coats were obtained from the Red Cross and then diluted with PBS in the ratio 1:1 and a portion was kept aside for **CD3 depletion** and -80°C storage. The rest were used in **density gradient centrifugation with Biocol** and PBMCs extracted. These were allowed to rest for one day before being stimulated with EBV peptide pools and then an **IFNγ secretion assay** (see next page) was done to obtain EBV specific T-cells. These cells were cultured along with irradiated unlabelled (negative) fraction of cells in material cup inserts or culture plates depending on the experiment. The culture was carried out for 18 days and feeding was done using **IL-2 supplemented medium**. After this the cells were harvested, **counted** and **analysed by flowcytometry**.

Continued next page...

EBV peptide stimulation protocol continued...

Interferon gamma secretion assay

EBV specific T-cells were generated from PBMCs with the use of an "Interferon γ (IFN-γ) secretion assay" which was provided by Miltenyi Biotech. The technique used in this assay relies on the fact that T-cells specific to EBV virus start to secrete IFN-γ on exposure to the virus peptide. Hence, the procedure consists of three stages:

i. Application of an "**IFN-γ catch reagent**" consisting of anti-IFN-γ monoclonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a).
ii. Application of "**IFN-γ detection antibody**" which is an anti-IFN-γ monoclonal antibody (mouse IgG1) conjugated to PE (R-phycoerythrin).
iii. Finally, "**anti-PE micro beads**" which are colloidal super-paramagnetic micro-beads conjugated to monoclonal mouse anti-PE antibody (mouse IgG1)

After PBMCs are extracted from buffy coats (please see: *"PBMC sources"* and *"Biocol separation of PBMCs from buffy coats or fresh blood"*) they are allowed to rest for one day and stimulated with EBV peptide pools (please see: *"preparation of EBV peptides"*). The stimulation is done with an EBV peptide concentration of 0.1 µg /ml in the presence of complete medium. The cells are incubated for 6 hours at 37°C. Next they are harvested with cold medium to stop the reaction and cells are counted. The first reagent (**IFN-γ catch reagent**) is added (25 µl of IFN-γ catch reagent + 100µl of "cold" medium for each 20 x 10^6 cells) and incubated for 5 minutes on ice.

Next the cells are incubated under slow continuous rotation along with warm medium at 37°C for 50 minutes. After incubation they are plunged in ice until the tubes are cooled in order to stop cellular secretion of IFN-γ. They are washed and the next reagent is added (**IFN-γ detection antibody**) in the same concentration per cell as the first reagent. This time the cells are incubated on ice for 10 minutes and in the dark.

The cells are again washed and the final reagent (**anti-PE micro beads**) is added again in the same concentration per cell as the previous two reagents. The mixture is incubated for 25 minutes at 4°C in the dark. The cells are washed and re-suspended in cold complete medium. A small aliquot is taken to stain for flowcytometry (precursor) to identify the overall population of IFN- γ secreting cells present in PBMCs.

The next steps are done using MS columns which are magnetic separation columns. These are columns provided by the same manufacturer of the IFN-γ secretion assay (Miltenyi Biotech). They consist of micro-beads which are extremely small, superparamagnetic particles capable of applying a high-gradient magnetic field. This is convenient to attract cells already labelled with anti-PE micro beads and hence provide a positive selection of T-cells secreting IFN-γ in response to EBV peptide pools. The cell suspensions are passed through two such columns and unlabelled cells are collected at the bottom of the columns. The unlabelled consist of cells not specific to EBV virus and are later taken for irradiation (30 gray) and used as feeder cells in culture (10 million per well in a 24-well plate).

The positive cells trapped inside the column are eluted into a culture dish (24-well plate) along with 1ml of complete medium supplemented with Interleukin-2 (IL-2). The eluted fraction was variable in cell number and normally dependant on the donor. Per column the number of EBV specific T-cells eluted was from 0.1 to 0.7 million cells.

When using this method of T-cell generation, the culture was carried out for 18 days and cells harvested in the end. Cell numbers were counted (please see "*Counting of cell number*"), stained and analysed (please see "*Staining and flowcytometry*").

b. Pan T-cell stimulation protocol *(for fresh blood sources)*:

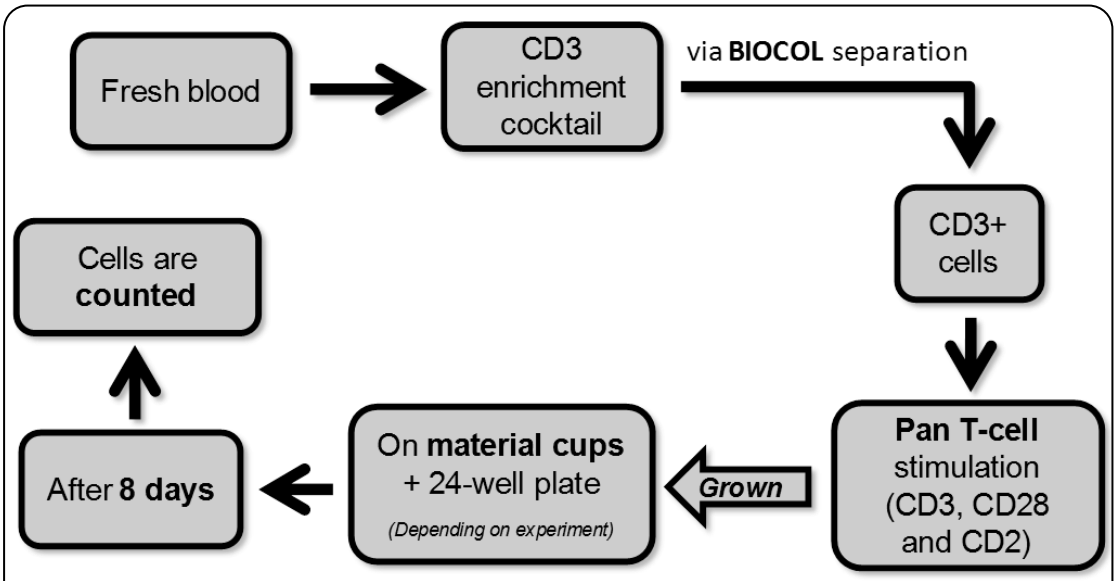


Figure 8.1a: Pan T-cell stimulation protocol.

*Description in brief (Please see other sections in "methods" for detailed explanations of each step in **bold**):*

Fresh blood was obtained from healthy donors and exposed to **CD3 enrichment cocktail** before performing a **density gradient centrifugation with Biocol**. Once T-cells (CD3+) cells were obtained they were further stimulated using **MACS iBead particles** (pan T-cell stimulation) which were previously prepared according to the manufacturer's instructions. The **T-cells along with MACS iBeads** (see below) are cultured in cups or plates depending on the experiment and after 8 days the cells are **counted**.

T-cell generation with MACS iBead particles

The MACS iBead particles are prepared as described earlier (please see "*Preparation of MACS iBead particles*"). T-cells are obtained from fresh blood after CD3 enrichment (please see "*CD3 enrichment*" next page). They are counted and re-suspended in complete medium at a concentration of 5 million cells per 900 µl of complete medium. The MACS iBead particles are taken in a tube at a concentration of 25 µl (2.5 million particles) per 5 million cells along with 200 µl complete medium and centrifuged at 300 g for 5 minutes.

The supernatant is aspirated and pellet broken after which it is re-suspended in a concentration of 100 µl per 5 million cells with complete medium. Next 100 µl is added to T-cell suspensions to make 1 ml out of each 900 µl suspensions. This is mixed well so that the particles are distributed evenly among the cells. The T cells are incubated at 37 °C and 5% CO_2 for 8 days and growth is observed. After 8 days of culture the cells are harvested and counted (please see "Counting *of cell number*")

5. CD3 depletion and freezing

When using buffy coats (as source of PBMCs) a portion of it was kept aside for CD3 depletion before carrying out Biocol extraction. This was done using a special kit called "human CD3 depletion cocktail" (Rosette-Sep®) which was applied to buffy coats according to the manufacturer's instructions (stem-cell technologies). Volume of buffy coat used for CD3 depletion depended on the size and extent of the experiment (10 to 20 ml).

The buffy coat was diluted with PBS in the ratio 1:1 and CD3 depletion cocktail was added in the concentration of 30µl per ml of buffy coat (as per manufacturer's instructions). This was done in the dark and incubation was carried out for 20 minutes at room temperature. After incubation the mixture was diluted with PBS in the ratio 1:1 and normal Biocol extraction carried out (please see: "*Biocol separation of PBMCs from buffy coats or fresh blood*", page 102)

6. CD3 enrichment

When using fresh blood from healthy donors (as source of PBMCs), CD3 enrichment was carried out using a special kit called "human CD3 enrichment cocktail" (Rosette-Sep®) which was applied to blood according to the manufacturer's instructions (stem-cell technologies).

The blood from the patient was collected (20 ml usually) and CD3 enrichment cocktail was added at 50µl per ml of whole blood (as per manufacturer's instructions). This was done in the dark and the mixture was incubated for 20 minutes at room temperature. After this PBS was used to dilute 1:1 and normal Biocol separation was carried out (please see: "*PBMC sources*" and "*Biocol separation of PBMCs from buffy coats or fresh blood*" above).

7. Culture

In most of the experiments unless stated otherwise, cell culture was carried out in 24-well plates. These are plates obtained from Becton Dickinson and are tissue culture treated by vacuum gas plasma.

Once T-cells were eluted after IFN-γ secretion assay, they were not disturbed for around 5 days with only occasional change of medium if turned yellow (half of old medium removed and fresh medium added to make up volume height of 1ml). Cultures were observed daily after this period and depending on medium colour, confluency and cell clumps (size and

colour), a decision was made to either split the culture into two wells or maintain the same culture.

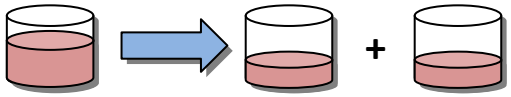


Figure 8.2: T cell culture splitting.

Splitting was done using a Pasteur pipette to mix cells and half was added to a new well as in figure 8.2. Fresh medium was added to make the volume height per well to 1 cm. Incubator settings used were 37^{o}C and 5% CO_2. harvesting was again done using a Pasture pipette after 18 days in the case of T-cells stimulated with EBV peptides and 8 days in the case of T-cells stimulated with MACS iBead particles. Extra medium was also added to emptied wells to extract any remaining cells sticking to the floor of the well.

8. Complete medium and IL-2

Medium preparation was as follows for all experiments (complete medium):

Table 8.2: Complete medium composition

Reagent	Volume (ml)
1645 RPMI (Biochrome)	500
10% FCS (foetal calf serum)	50
5% Penstrep (Penicillin / Streptomycin (Biochrome))	5

When feeding cells during cell culture complete medium was combined with Interleukin-2 (rhIL-2) at 100 units per ml of complete medium.

9. Counting of Cell number

Counting was done using Tryptan blue and Countess® Automated Cell Counter from Invitrogen. Cell samples used for counting were 10 µl which was combined with 10 µl of Tryptan blue provided by the manufacturer. The operation of the device was as per manufacturer's instructions. Fold expansion was calculated by dividing the final cell number (day 18) with the initial cell number (day 0).

10. Staining and Flowcytometry

Once culture was carried out for 18 days the T-cells were harvested, counted and stained for surface and intracellular markers. The aim of the staining was to determine the phenotype, functionality and maturation status of all the cells after culture.

The procedure for staining is as follows:

PBMCs were previously CD3 depleted and frozen in -80 °C storage (please see "*CD3 depletion and freezing*" above). These are thawed out and re-suspended in complete medium and allowed to rest for one day. Next day they are counted and total number divided as needed for T-cell stimulation (10 PBMCs: 1 T-cell).

Three types of stimulation are used for each sample being tested:

1. DMSO: PBMCs are combined with T-cells along with 6 µl of 90% DMSO (used as negative control)
2. PMA/IONO: T-cells are stimulated with a mixture of PMA / IONO (used as positive control)
3. EBV peptides: The PBMCs are exposed to EBV peptides for 1 hour before using them to stimulate T-cells. Peptides are used at the concentration of 1 µg per ml of complete medium.

The above three are incubated for 1 hour at 37 °C in the slant position in the presence of 1µl of Monensin (Becton Dickinson) and 20µl of anti-CD107a (PE) (Becton Dickinson). Next Brefeldine-A (BFA) (Sigma-Aldrich) is added at 2.5 µg per ml of complete medium and incubated for 4 hours at 37 °C.

After this the cells are washed with FACS buffer (PBS with 5% FCS) and stained with the following extracellular stains:

Table 8.3: Surface markers and maturation stains

CD3 – Percp (Becton Dickinson)	**10µl**
CD4 – PE-Cy7 (Becton Dickinson)	**3µl**
Live Dead – APC-CY7 (Invitrogen)	**0.5µl**

Stains for maturation status

CD 45 RA – ECD (Beckman Coulter)	**1.5 µl**
CCR7 * - un-conjugated (R & D Systems)	1 **µl**

*** CCR7** is stained by secondary antibody: First 1µl anti-human CCR7 mouse antibody (R & D Systems) is added and next 1µl **goat anti-mouse antibody** (Southern Biotech) and finally 1µl **Streptavidin –Pacific blue** (Invitrogen) with washing steps in-between.

Permeabilization is done using Perm2 BD solution (BD Biosciences) according to the manufacturer's instructions and intracellular staining is done next using the following stains:

Table 8.4: Stains for cytokine secretion and CD8

IFNγ – Alexa700 (Becton Dickinson)	**1µl**
TNFα – FITC (Becton Dickinson)	**0.5 µl**
CD8 – APC (Becton Dickinson)	**3µl**

After this the cells are washed again and measured at the **BD™ LSR II flowcytometer** and analysis was carried out with the data obtained.

11. Geometry testing (plates + approaches)

In the geometry testing experiments, three types of culture plates were used all made of surface treated polystyrene and all having circular shaped culture chambers as shown in the schematic in figure 8.3.

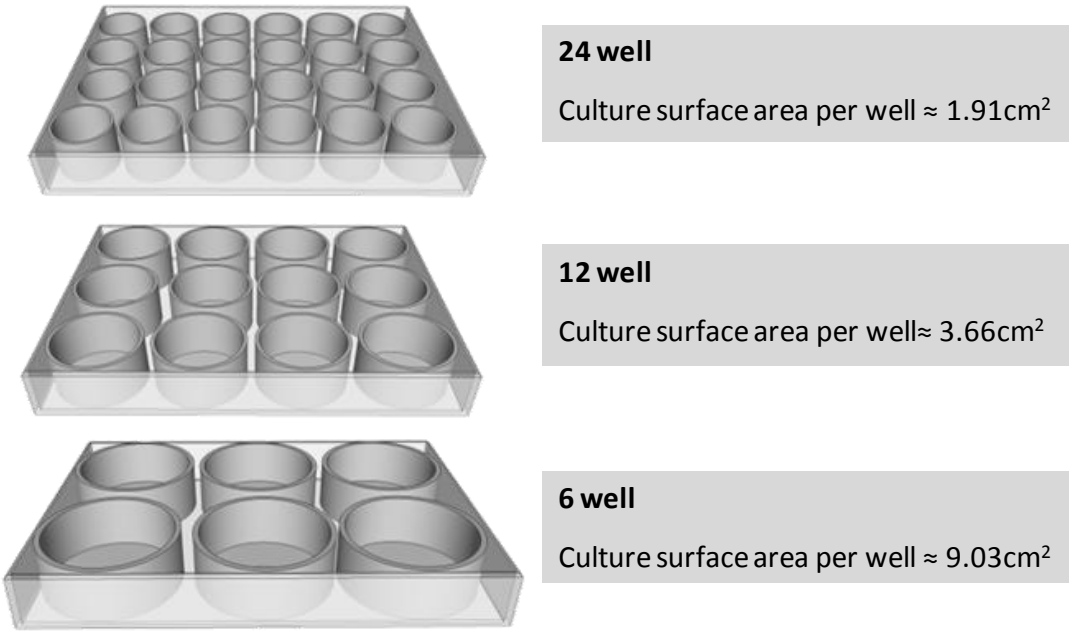


Figure 8.3: Culture plates with respective culture surface area per well. Medium height was maintained at approximately 1 cm throughout the culture period.

During culture in these plates the medium height was maintained at approximately 1 cm, so the only variables changing between plates were surface area and volume. An increase in culture surface area occurred when moving from a 24 to a 12 and finally 6-well plate which

had the largest culture surface area. Volume too increased in a similar manner and proportional to the surface area when medium height was maintained at 1 cm.

Table 8.5: Volume and surface area per well of plates used in geometry testing experiments

Plate	Culture surface area per well (cm^2)	Radius of culture surface per well (cm)	Volume per well cm^3 (1 cm height)
24 well plate	1.91	0.779725517	1.91
12 well plate	3.66	1.079358228	3.66
6 well plate	9.03	1.695387352	9.03

Next different approaches were planned so that a gradual or sudden change in geometry could be compared to the normal 24-well plate culture as shown in figure 8.4.

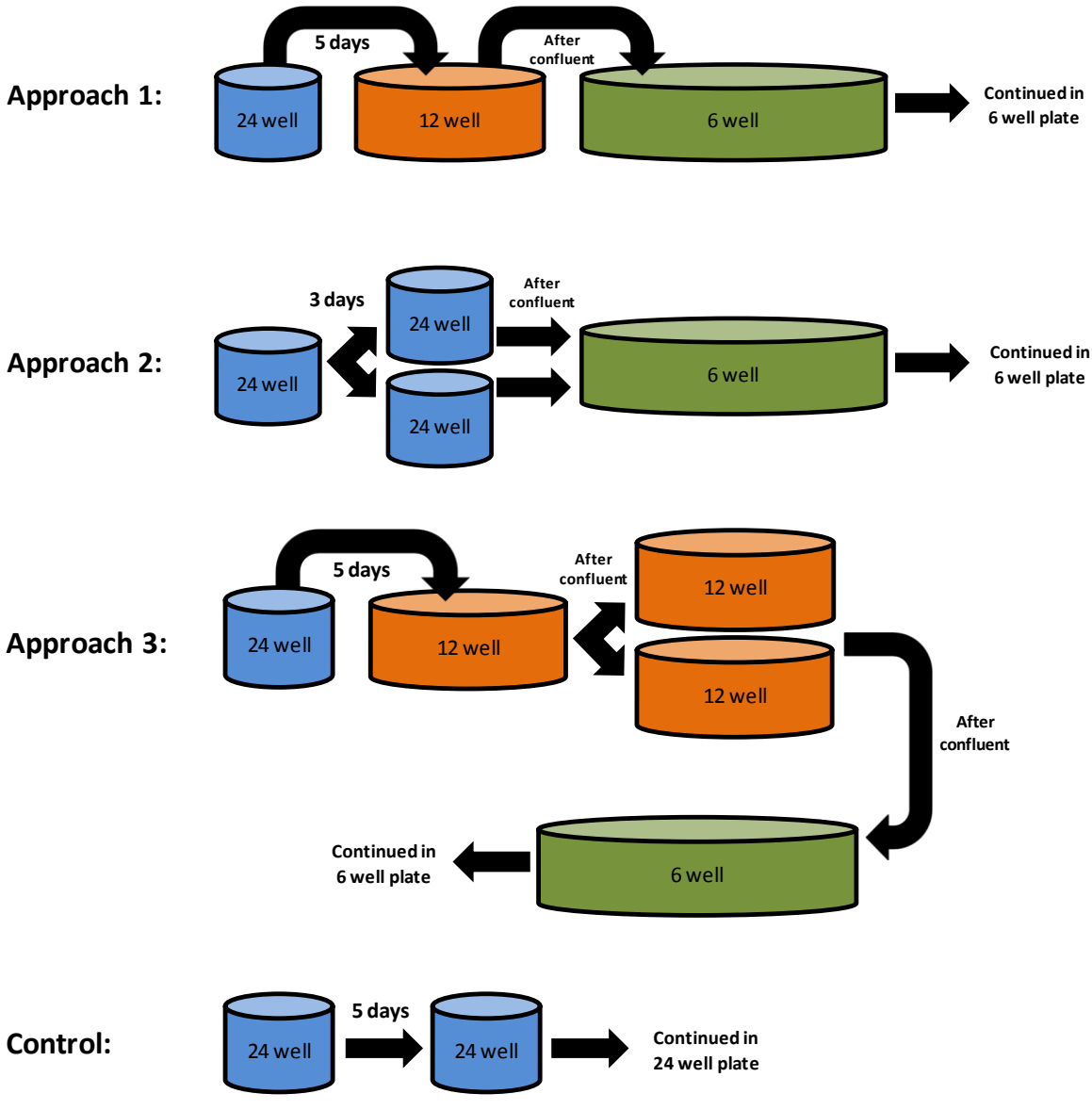


Figure 8.4: Approaches used in geometry testing experiments.

There is a gradual change in geometry in **approach 1** where culture is started in a single 24-well and after 5 days moved to a single 12-well and after confluence to a 6-well. This approach is to test if T-cells prefer a gradual increase in geometry size during the initial stages of culture.

Next **approach 2** is a sudden change in geometry where in 3 days the cells are split from the first 24-well in to two 24-wells and next into a 6-well once confluent. **Approach 3** is again a gradual change in geometry but it is much more gradual than **approach 1** where the 12-well is split into two 12-wells before moving into a 6-well. These approaches were compared to the **control** (normal 24-well plate culture) where the geometry is not changing and culture is started in a single 24-well and split after 5 days and continued in 24-wells.

12. Material cup inserts and roughness

Material testing experiments on T-cells was done with the help of material cup inserts as shown in figure 8.5 and 8.6:

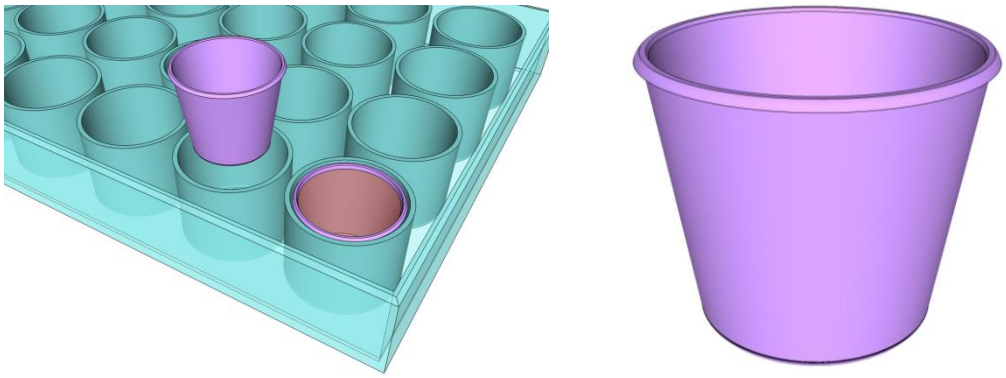


Figure 8.5: Schematic of material cup inserts showing how they "sit" inside a regular 24-well plate. The second figure shows a single cup. Each cup is made entirely out of a single material by injection moulding.

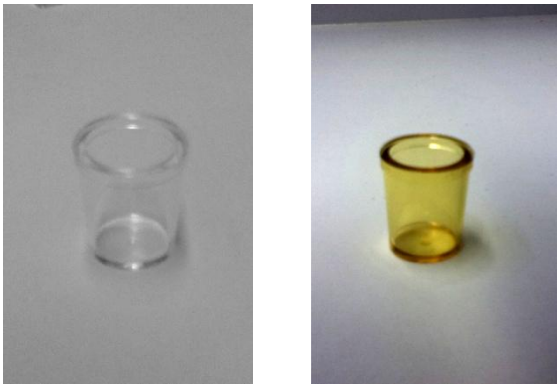


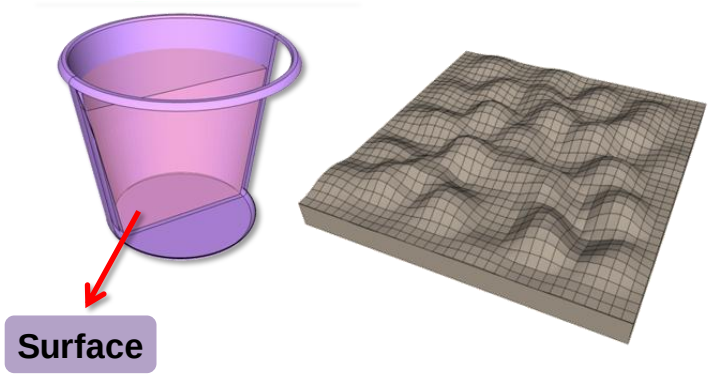
Figure 8.6: Examples of two kinds of material cups used.

These material cups were made entirely out of one kind of material through injection moulding in constant dimensions. This ensured that T-cells when grown inside these cups were surrounded by only a one material and were exposed to fixed geometries. The cups were designed in such a way that they would easily fit inside a regular 24-well plate. These cups were manufactured and supplied by the Helmholtz-Zentrum Geesthacht (HZG) Centre for Materials and Coastal Research, Berlin, Germany.

Table 8.6: Materials used in testing

Material	Notation
Polystyrene	PS
Polycarbonate	PC
Poly(styrene-co-acrylonitrile)	PSAN
Poly(ether imide)	PEI
Poly(etherurethane) or Tecoflex®	TFX

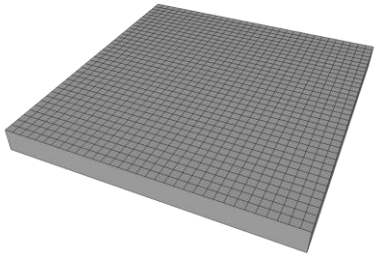
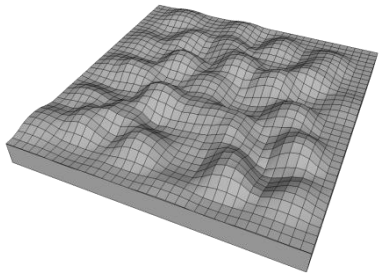
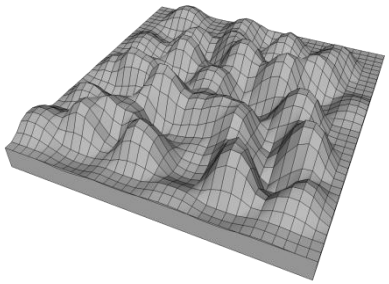
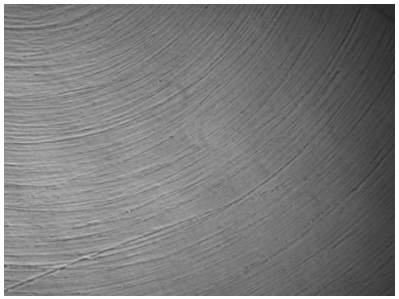
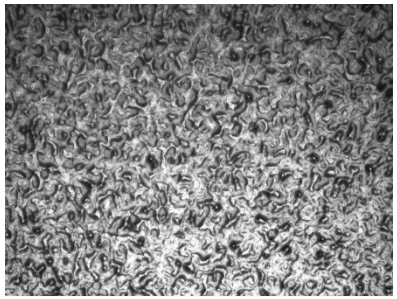
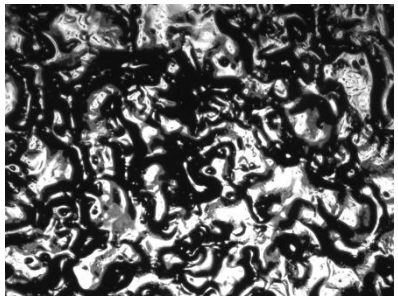
Additionaly the advantage of having material cups was that we could physically modify the culture surface in terms of roughness as in figure 8.7.



TFigure 8.7: Rough surface modification in material cups.

This was done with the help of stamps which had fixed patterns that was imparted to the culture surface of the cups during injection moulding. This ensured uniformity in pattern and grade of roughness. In our experiments cups with three grades of roughness (table 8.7, next page) were used and denoted as such: R00, R30 and R45. These names don't signify the quantity or quality of roughness; they were just used to represent the three grades of roughness used in our experiments.

Table 8.7: Three grades of roughness used in roughness testing experiments (R00, R30 and R45)

Schematic			
Without cells			
Nomenclature	**R00**	**R30**	**R45**
Ra values	Ra = 0.14 ± 0.05 µm	Ra = 2.68 ± 0.13 µm	Ra = 17.33 ± 0.23 µm

13. Electrospun material

Similar to the roughness modification we also used electrospun material at the culture surface of the cups figure 8.8. These are fibres produced from the same material used to construct the material cups. Two types of fibre alignment, parallel and random were used. These were used to expose T-cells to a different type of rough surface compared to the physical modified surfaces of material cups.

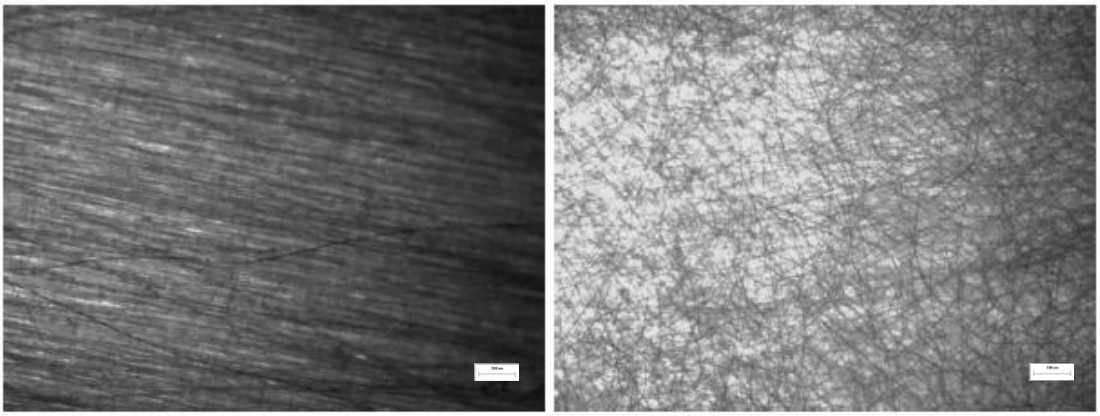


Figure 8.8: Electrospun material used for experiments as viewed under microscope before inoculating with cells.

14. Fixed splitting schedule

A fixed splitting schedule was formulated to split cells at fixed time points throughout an 18 day period of culture. The splitting time points were followed as shown in the table 4 without regard to the confluency of cells in each well. Culture always started with a single well in a 24-well plate and ended with 32 wells on day 18. Medium was fed depending on medium colour along with IL-2 (100 units per ml of complete medium).

Table 8.8: Fixed splitting schedule

Wells	Day	Date	Procedure done
1 well	0		
	1		
	2		
	3		
	4		
	5		
2	6		
	7		
	8		
4	9		
	10		
	11		
8	12		
	13		
16	14		
	15		
32	16		
	17		
	18		

15. Mixing table

The cell culture dissociation experiments were performed with the use of a mixing-table (Mixmate®) courtesy of Eppendorf. The speed (revolutions per minute) and time (minutes) could be set before starting the machine. The device can securely retain a regular culture plate on the top via an adapter as in figure 8.9 below.

Figure 8.9: Mixmate® from Eppendorf used for cell culture dissociation experiments. ***Image from http://www.eppendorf.co.uk/int/index.php?l=3&action=products&catalognode=31952&pb=1e41bfc6d0fd02cd on 25th January 2013***

16. G-Rex bioreactor (Wilson Wolf Manufacturing Corporation)

The G-Rex bioreactor was designed specifically for the purpose of T-cell culture [57]. It consists of a gas permeable membrane on the bottom that enables gas diffusion and also allows growth of cells on its surface making the cells have direct contact with the gases diffusing across the membrane. This permits large amounts of medium to be used in these bioreactors without worrying about gas supply to the cells.

Figure 8.9: G-Rex bioreactor. *Image taken from http://www.wilsonwolf.com/page/show/66809-g-rex-specification on 25th January 2013*

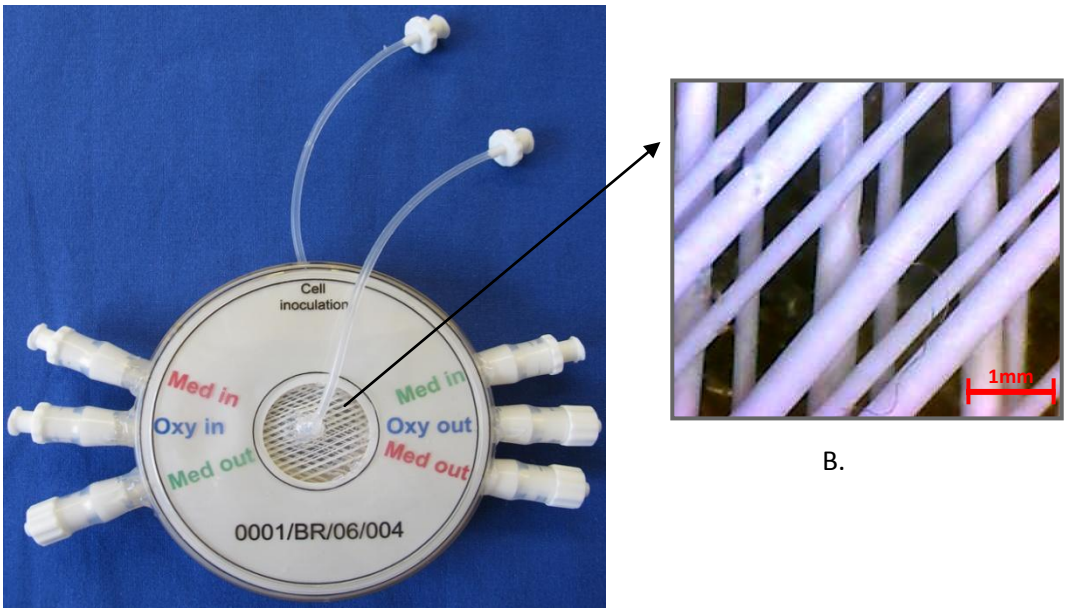
Expansion was carried out in the smallest version of the bioreactor shown in the figure 8.9 which had a culture surface area of 10 cm^2 and a 40 ml medium capacity.

T-cells were generated using the EBV peptide stimulation protocol (page 104) and the eluted cells were inoculated in the bioreactor along with 10 million feeder cells. These cells were cultured together for 18 days in this bioreactor and half of the medium was changed once in a week. The medium volume used was approximately 30 ml.

17. Perfusion bioreactor (Stem Cell Systems)

A perfusion bioreactor from Stem Cell Systems, Berlin, Germany was used in our experiments to culture T-cells in the system comparison experiments. The bioreactor had a central chamber with two layers of capillaries that delivered gas and medium to the inner contents of the chamber as in figure 8.10.

A.



B.

Figure 8.10: Schematic of the perfusion bioreactor. (a) Connections and bioreactor (b) Capillaries view from above. *Image courtesy of Dr. K. Zeilinger and research group at the Charité – Universitätsmedizin, Campus Virchow-Klinikum, Biomedical Research Centre, Berlin, Germany.*

Expansion was carried out in the central chamber (with capillaries) of the bioreactor shown in the figure 8.10 which had a culture surface area of 3.1 cm^2 and a 0.5 ml culture chamber volume. The culture chamber was connected to a perfusion system (tubing and pump system) which re-circulated the culture medium and was set at a flow rate of 3ml per minute. The total volume of complete medium flowing through this perfusion system was approximately 10 ml.

There was also a possibility to add fresh medium if required through a second perfusion system (pump and tubing) and this was used when samples were taken out of the system to supplement the volume of medium lost.

Operation of the bioreactor was carried out as close as possible to the normal 24-well plate culture conditions. Parameters were monitored by the computer and set as follows:

Temperature: 37°C

Air flow rate: 5 ml/min

Carbon dioxide concentration: 5%

T-cells were generated using the EBV peptide stimulation protocol (page 104) and the eluted cells were inoculated in the bioreactor along with 10 million feeder cells. These cells were cultured together for 18 days in this bioreactor.

harvesting of cells was done by cutting open the top cover of the central chamber using a saw (bi-metal 25 mm, garant, Hoffmann GmbH, Germany) and opening under a sterile hood.

18. Analysis

After staining T-cells (please see *"Staining and Flowcytometry"* page 110) they were analysed via the **BD™ LSR II flow cytometer**. Data obtained was analysed on Excel (Microsoft Corporation) and SPSS (IBM software).

19. Statistics

Data obtained from flowcytometry (FACS) was statistically analysed using the software **SPSS** (IBM software).

Normalcy was determined using the Shapiro-Wilk and Kolmogorov-Smirnov normality test. Non-parametric Wilcoxon matched-pairs test was used to calculate statistical significance in all studies (except for a few which was calculated using Mann-Whitney U test). Probability (p) values were considered significant if it was ≤ 0.05.

20. Graphs

Once data was collected after FACS and statistical analysis the results were plotted in graphs using either Excel (Microsoft office 2007) or Graph pad prism 6 (Graph pad software, Inc).

21. Materials / consumables used in all experiments

Reagents

Golgi Stop	Becton Dickinson, Heidelberg
Brefeldin A	Becton Dickinson, Heidelberg
Ionomycin	Sigma Aldrich, Schnelldorf
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, Schnelldorf
PBS buffer solution	PAA, Pasching, Austria
RPMI medium	Biochrom, Berlin
Fetal calf serum	Biochrom, Berlin
Penicillin-streptomycin solution	Biochrom, Berlin
Dimethylsulfoxide (DMSO)	Sigma Aldrich, Schnelldorf
Sodium azide (NaN_3) Solution	Merck, Darmstadt
FACS Perm-2 solution	Becton Dickinson, Heidelberg

Apparatus

Cryogenic freezing tubes	Greiner, Ammerbuch
24-well cell culture plates	Becton Dickinson, Heidelberg
12-well cell culture plates	Becton Dickinson, Heidelberg
6-well cell culture plates	Becton Dickinson, Heidelberg
Polystyrene material cup inserts (R00, R30, R45)	Helmholtz-Zentrum Geesthacht, Berlin
Polycarbonate material cup inserts (R00, R30, R45)	Helmholtz-Zentrum Geesthacht, Berlin
Poly(styrene-co-acrylonitrile) material cup inserts (R00, R30, R45)	Helmholtz-Zentrum Geesthacht, Berlin
Poly(ether imide) material cup inserts (R00, R30, R45)	Helmholtz-Zentrum Geesthacht, Berlin
Poly(etherurethane) / Tecoflex® material cup inserts (R00, R30, R45)	Helmholtz-Zentrum Geesthacht, Berlin
3.5 ml transfer pipettes	Sarstedt, Nümbrecht
5 , 10 , 25 ml disposable pipettes	Becton Dickinson, Heidelberg
10, 100, 1000 µl pipette tips	Eppendorf, Hamburg
15 and 50 ml falcon tubes	Becton Dickinson, Heidelberg
FACS tubes	Becton Dickinson, Heidelberg
G-Rex bioreactor	Wilson Wolf Manufacturing Corporation, New Brighton, USA
Perfusion bioreactor	Stem Cell Systems, Berlin

Media and buffers

Cell culture medium	500ml RPMI medium, 2 mM L-glutamine, 10% FCS, 1% penicillin / streptomycin
T-cell culture medium	500ml RPMI medium, 2 mM L-glutamine, 10% FCS, 1% penicillin / streptomycin, 100 IU / ml IL-2
Freezing	50ml FCS, 10% DMSO
FACS buffer	500ml PBS, 1% FCS, 0.01% NaN_3

Antibody

Anti-human CD3 (PerCP)	Becton Dickinson, Heidelberg
Anti-human CD4 (PE-Cy7)	Becton Dickinson, Heidelberg
Anti-human CD8 (APC)	Becton Dickinson, Heidelberg
Anti-human CD16 (FITC)	Becton Dickinson, Heidelberg
Anti-human CD45RA (ECD)	Beckman Coulter, Krefeld
Antihuman CD107a (PE)	Becton Dickinson, Heidelberg
Anti-human CCR7 (un-conjugated)	R & D Systems, Wiesbaden-Nordenstadt
Goat anti-Mouse IgG2a, human ads-Biot	Biozol, Eching
Anti-human IFN (Alexa700)	Becton Dickinson, Heidelberg
Live / Dead APC Cy7	Invitrogen Camarillo, Canada
Anti-human TNFa (FITC)	Becton Dickinson, Heidelberg
RosetteSep CD3 Depletion Kit	StemCell Technologies, Grenoble, France
RosetteSep CD3 Enrichment Kit	StemCell Technologies, Grenoble, France
Streptavidin (Pacific Blue)	Invitrogen Camarillo, Canada

Magnetic cell separation

IFNg secretion assay (cell enrichment and detection kit)	Miltenyi Biotec, Bergisch Gladbach
MACS separation columns (MS, LS)	Miltenyi Biotec, Bergisch Gladbach
MACS Multistand	Miltenyi Biotec, Bergisch Gladbach
MACSmix Rotator	Miltenyi Biotec, Bergisch Gladbach

Cell separation using Anti-Biotin MACSiBead Particles

T Cell Activation/Expansion Kit, human	Miltenyi Biotec, Bergisch Gladbach

Devices

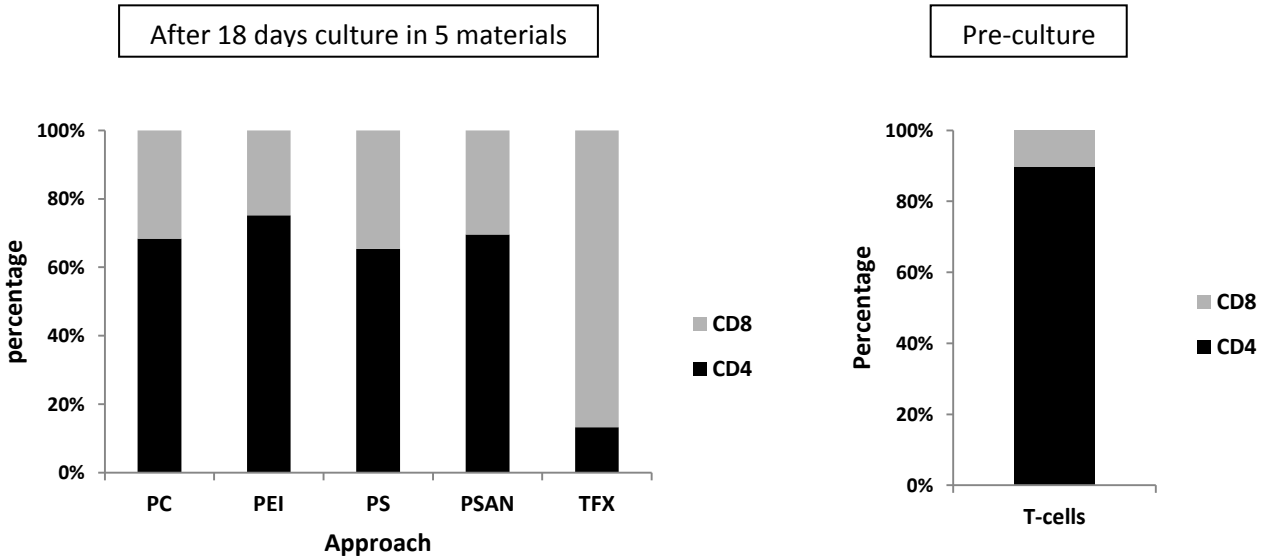
Irradiation appliance Gammacell-40	Atomic Energy, Mississauga, Canada
LSR II flow cytometer	Becton Dickinson, Heidelberg
Freezer (-80 ° C)	Liebherr, Oberhausen
Incubator	Heraeus, Berlin
Refrigerator	Liebherr, Oberhausen
Microscope	Leica, Brunswick
Axiovert 40 CFL Microscope (Photography)	Carl Zeiss Microscopy, U.S.A
High Content Screener (Operetta system)	PerkinElmer Inc., Massachusetts, U.S.A
Clean bench	Heraeus, Berlin
Nitrogen tank	Taylor-Wharton, Husum
Freezing container	Nalgene, Roskilde, Denmark
Waterbath	GFL, Burgwedel
Centrifuge	Biofuge, Heraeus, Berlin
Countess® Automated Cell Counter	Invitrogen, Camarillo, Canada
Mixmate®	Eppendorf, UK

Software

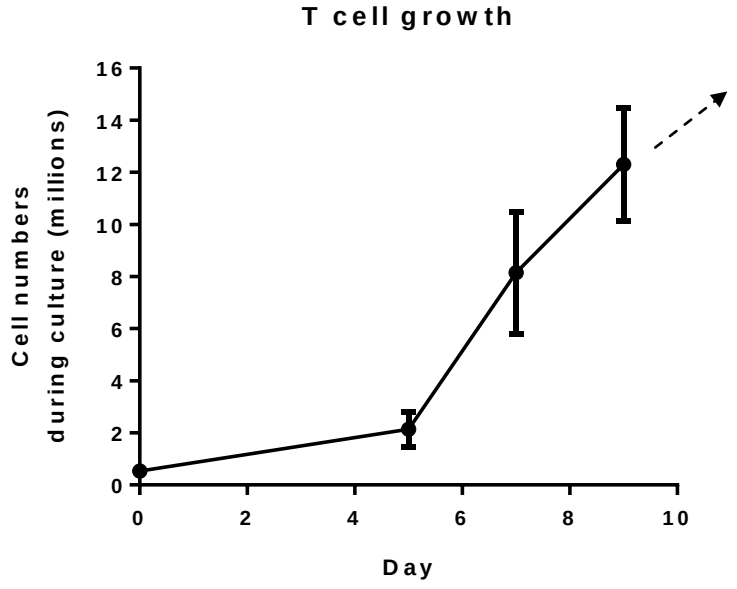
GraphPad Prism 6	GraphPad Software
SPSS statistics	IBM Software
Sketch-up 8	Trimble Navigation Ltd
Microsoft Office 2007	Microsoft corporation
Flow Jo	Tree Star Inc
BD FACSDiva Software	BD Biosciences
Columbus (Image Data Storage and Analysis System)	PerkinElmer Inc.
Cell explorer (Automation Platform)	PerkinElmer Inc.

Appendix

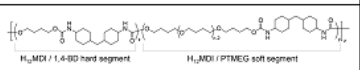
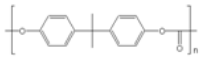
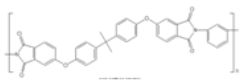
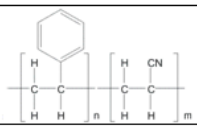
<u>Figure 1:</u> CD4 and CD8 proportions before and after culture on TFX material.
T-cells generated from buffy coats were cultured on 5 materials (PS, PC, PSAN, PEI and TFX) with smooth surfaces (R00) using the EBV peptide stimulation protocol and CD4 and CD8 T-cell proportions were determined on day 0 before culture and after 18 days of culture. The proportion of CD4 and CD8 appears affected in T-cells grown on TFX but was minimally affected when grown on other materials.

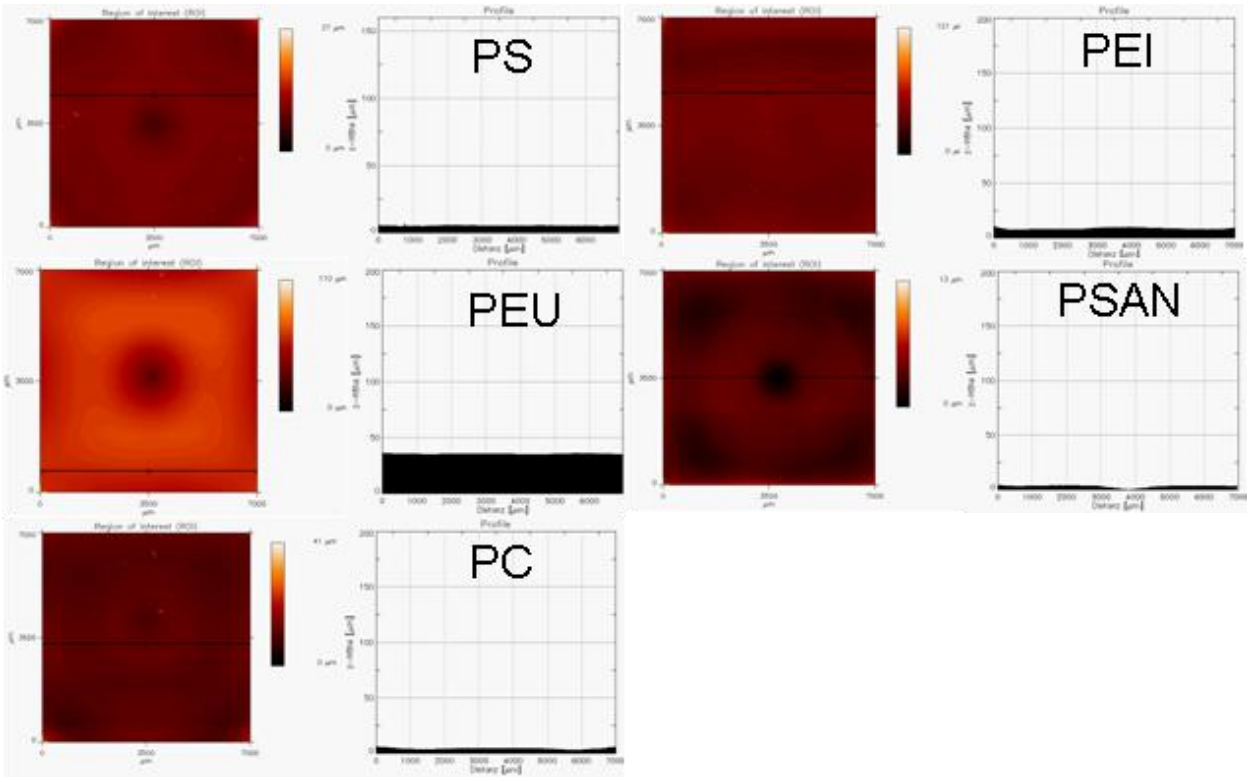


<u>Figure 2:</u> T-cell proliferation during the initial stages of culture.
T-cells generated from fresh blood were cultured from three healthy donors (n = 3) using the EBV peptide stimulation protocol and cell numbers determined at days 0, 5, 7 and 9. The rate of expansion appears to be exponential after day 5 or 6. Values shown are mean values with standard deviation.



<u>Figure 3:</u> Specifications of the five materials tested in our study.
These specifications were kindly donated by the people at the Helmholtz-Zentrum Geesthacht; Berlin, Germany. The material Tecoflex™ is also known as poly(etherurethane) PEU.

material			T_g/T_m [° C]	E [MPa]	M_n [kg/mol]	Rq [µm]	θadv [°]	θrec [°]
polystyrene	PS		103.6-106.9	129.3 ±77.8	109	0.12 ± 0.04	98,9 ± 5,0	79,1 ±11,6
poly(etherurethane)	PEU		55.9	401.1 ±24.8	61.1	0.863 ± 0.07	84,0 ± 7,1	57,7 ± 8,2
polycarbonate	PC		149.2-150.0	122.4 ±52.4	17.7	0.335 ± 0.13	85,0 ± 7,9	68,2 ±3,9
pooly(ether imide)	PEI		215.2	178.6 ±46.0	16.8	0.23 ± 0.07	92,8 ± 2,6	66,8 ± 3,6
poly(styrene-co-acrylonitrile)	PSAN		107.6	156.9 ±38.5	67.9	0.256 ± 0.07	87,1 ± 5,3	68,7 ± 4,2



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Bibliography:

1. Bollard, C.M., Rooney, C.M. & Heslop, H.E. T-cell therapy in the treatment of post-transplant lymphoproliferative disease. *Nat Rev Clin Oncol* 9, 510-519.
2. Pagliara, D. & Savoldo, B. Cytotoxic T lymphocytes for the treatment of viral infections and posttransplant lymphoproliferative disorders in transplant recipients. *Current opinion in infectious diseases* 25, 431-437.
3. Sellar, R.S. & Peggs, K.S. Therapeutic strategies for the prevention and treatment of cytomegalovirus infection. *Expert opinion on biological therapy* 12, 1161-1172.
4. Bohnenkamp, H., Hilbert, U. & Noll, T. Bioprocess development for the cultivation of human T-lymphocytes in a clinical scale. *Cytotechnology* 38, 135-145 (2002).
5. DiGiusto, D. & Cooper, L. Preparing clinical grade Ag-specific T cells for adoptive immunotherapy trials. *Cytotherapy* 9, 613-629 (2007).
6. Atkuri, K.R., Herzenberg, L.A. & Herzenberg, L.A. Culturing at atmospheric oxygen levels impacts lymphocyte function. *Proceedings of the National Academy of Sciences of the United States of America* 102, 3756-3759 (2005).
7. Gerard, A. *et al.* Secondary T cell-T cell synaptic interactions drive the differentiation of protective CD8+ T cells. *Nature immunology* 14, 356-363.
8. Carswell, K.S. & Papoutsakis, E.T. Culture of human T cells in stirred bioreactors for cellular immunotherapy applications: shear, proliferation, and the IL-2 receptor. *Biotechnology and bioengineering* 68, 328-338 (2000).
9. Fu, Y. *et al.* A novel mechanism of gamma/delta T-lymphocyte and endothelial activation by shear stress: the role of ecto-ATP synthase beta chain. *Circulation research* 108, 410-417.
10. Cooper, D., Pride, M.W., Brown, E.L., Risin, D. & Pellis, N.R. Suppression of antigen-specific lymphocyte activation in modeled microgravity. *In vitro cellular & developmental biology* 37, 63-65 (2001).
11. Hislop, A.D., Taylor, G.S., Sauce, D. & Rickinson, A.B. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annual review of immunology* 25, 587-617 (2007).
12. Steven, N.M. *et al.* Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *The Journal of experimental medicine* 185, 1605-1617 (1997).
13. Barrett, L., Fowke, K.R. & Grant, M.D. Cytomegalovirus, aging, and HIV: a perfect storm. *AIDS reviews* 14, 159-167.
14. Fujita, Y., Rooney, C.M. & Heslop, H.E. Adoptive cellular immunotherapy for viral diseases. *Bone marrow transplantation* 41, 193-198 (2008).
15. Bollard, C.M. *et al.* Good manufacturing practice-grade cytotoxic T lymphocytes specific for latent membrane proteins (LMP)-1 and LMP2 for patients with Epstein-Barr virus-associated lymphoma. *Cytotherapy* 13, 518-522.
16. Sili, U. *et al.* Production of good manufacturing practice-grade cytotoxic T lymphocytes specific for Epstein-Barr virus, cytomegalovirus and adenovirus to prevent or treat viral infections post-allogeneic hematopoietic stem cell transplant. *Cytotherapy* 14, 7-11.
17. Bollard, C.M. *et al.* Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *The Journal of experimental medicine* 200, 1623-1633 (2004).
18. Roskrow, M.A. *et al.* Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood* 91, 2925-2934 (1998).
19. Juvet, S.C. & Zhang, L. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. *Journal of molecular cell biology* 4, 48-58.
20. Godfrey, D.I., Hammond, K.J., Poulton, L.D., Smyth, M.J. & Baxter, A.G. NKT cells: facts, functions and fallacies. *Immunology today* 21, 573-583 (2000).
21. Rodriguez, A. & Anderson, J.M. Evaluation of clinical biomaterial surface effects on T lymphocyte activation. *J Biomed Mater Res A* 92, 214-220.
22. Eriksson, C., Lausmaa, J. & Nygren, H. Interactions between human whole blood and modified TiO2-surfaces: influence of surface topography and oxide thickness on leukocyte adhesion and activation. *Biomaterials* 22, 1987-1996 (2001).
23. de los Angeles Aguilar, M., Espinosa, S., Rodriguez, L. & Pina, C. Biocompatibility in vitro tests of Zinalco. *Mutation research* 446, 129-134 (1999).

24. Stea, S. *et al.* In vitro sister chromatid exchange induced by glass ionomer cements. *J Biomed Mater Res* 40, 545-550 (1998).
25. Ikada, Y. Surface modification of polymers for medical applications. *Biomaterials* 15, 725-736 (1994).
26. Amiji, M. & Park, K. Prevention of protein adsorption and platelet adhesion on surfaces by PEO/PPO/PEO triblock copolymers. *Biomaterials* 13, 682-692 (1992).
27. Courtney, J.M., Lamba, N.M., Sundaram, S. & Forbes, C.D. Biomaterials for blood-contacting applications. *Biomaterials* 15, 737-744 (1994).
28. Gretz, J.E., Anderson, A.O. & Shaw, S. Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex. *Immunological reviews* 156, 11-24 (1997).
29. Itescu, S., Ankersmit, J.H., Kocher, A.A. & Schuster, M.D. Immunobiology of left ventricular assist devices. *Progress in cardiovascular diseases* 43, 67-80 (2000).
30. Schuster, M. *et al.* Induction of T-cell apoptosis by polyurethane biomaterials used in left ventricular assist devices is dependent on calcineurin activation. *Transplantation proceedings* 33, 1958-1959 (2001).
31. Schuster, M. *et al.* B-cell activation and allosensitization after left ventricular assist device implantation is due to T-cell activation and CD40 ligand expression. *Human immunology* 63, 211-220 (2002).
32. Yoshii, T., Dumas, J.E., Okawa, A., Spengler, D.M. & Guelcher, S.A. Synthesis, characterization of calcium phosphates/polyurethane composites for weight-bearing implants. *Journal of biomedical materials research* 100, 32-40.
33. Vanhoutte, V.J. *et al.* Cytolytic mechanisms and T-cell receptor Vbeta usage by ex vivo generated Epstein-Barr virus-specific cytotoxic T lymphocytes. *Immunology* 127, 577-586 (2009).
34. Scherrenburg, J., Piriou, E.R., Nanlohy, N.M. & van Baarle, D. Detailed analysis of Epstein-Barr virus-specific CD4+ and CD8+ T cell responses during infectious mononucleosis. *Clinical and experimental immunology* 153, 231-239 (2008).
35. Ning, R.J., Xu, X.Q., Chan, K.H. & Chiang, A.K. Long-term carriers generate Epstein-Barr virus (EBV)-specific CD4(+) and CD8(+) polyfunctional T-cell responses which show immunodominance hierarchies of EBV proteins. *Immunology* 134, 161-171.
36. Gooden, M.J., de Bock, G.H., Leffers, N., Daemen, T. & Nijman, H.W. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *British journal of cancer* 105, 93-103.
37. Sun, J.C., Williams, M.A. & Bevan, M.J. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nature immunology* 5, 927-933 (2004).
38. Rooney, C.M. *et al.* Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92, 1549-1555 (1998).
39. Matloubian, M., Concepcion, R.J. & Ahmed, R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *Journal of virology* 68, 8056-8063 (1994).
40. Cardin, R.D., Brooks, J.W., Sarawar, S.R. & Doherty, P.C. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *The Journal of experimental medicine* 184, 863-871 (1996).
41. Flinsenberg, T.W., Compeer, E.B., Boelens, J.J. & Boes, M. Antigen cross-presentation: extending recent laboratory findings to therapeutic intervention. *Clinical and experimental immunology* 165, 8-18.
42. Fooksman, D.R. *et al.* Functional anatomy of T cell activation and synapse formation. *Annual review of immunology* 28, 79-105.
43. Takada, K. & Jameson, S.C. Naive T cell homeostasis: from awareness of space to a sense of place. *Nature reviews* 9, 823-832 (2009).
44. Sprent, J. & Surh, C.D. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. *Nature immunology* 12, 478-484.
45. Coombs, D. & Goldstein, B. Effects of the geometry of the immunological synapse on the delivery of effector molecules. *Biophysical journal* 87, 2215-2220 (2004).
46. Miranti, C.K. & Brugge, J.S. Sensing the environment: a historical perspective on integrin signal transduction. *Nature cell biology* 4, E83-90 (2002).
47. Shimizu, Y. LFA-1: more than just T cell Velcro. *Nature immunology* 4, 1052-1054 (2003).

48. Kannanganat, S., Ibegbu, C., Chennareddi, L., Robinson, H.L. & Amara, R.R. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *Journal of virology* 81, 8468-8476 (2007).
49. Ley, K., Laudanna, C., Cybulsky, M.I. & Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature reviews* 7, 678-689 (2007).
50. Ellingsen, T. *et al.* Migration of mononuclear cells in the modified Boyden chamber as evaluated by DNA quantification and flow cytometry. *Scandinavian journal of immunology* 52, 257-263 (2000).
51. Yewdell, J.W. & Haeryfar, S.M. Understanding presentation of viral antigens to CD8+ T cells in vivo: the key to rational vaccine design. *Annual review of immunology* 23, 651-682 (2005).
52. Foster, A.E. *et al.* Large-scale expansion of cytomegalovirus-specific cytotoxic T cells in suspension culture. *Biotechnology and bioengineering* 85, 138-146 (2004).
53. Simons, D.M., Gardner, E.M. & Lelkes, P.I. Dynamic culture in a rotating-wall vessel bioreactor differentially inhibits murine T-lymphocyte activation by mitogenic stimuli upon return to static conditions in a time-dependent manner. *J Appl Physiol* 100, 1287-1292 (2006).
54. Tran, C.A. *et al.* Manufacturing of large numbers of patient-specific T cells for adoptive immunotherapy: an approach to improving product safety, composition, and production capacity. *J Immunother* 30, 644-654 (2007).
55. Ryo Ohashi, D.V.S., Jean-François P. Hamel *Perfusion Cell Culture in Disposable Bioreactors*, Vol. 1. (Springer Netherlands, 2001).
56. Sadeghi, A. *et al.* Large-scale bioreactor expansion of tumor-infiltrating lymphocytes. *Journal of immunological methods* 364, 94-100.
57. Vera, J.F. *et al.* Accelerated production of antigen-specific T cells for preclinical and clinical applications using gas-permeable rapid expansion cultureware (G-Rex). *J Immunother* 33, 305-315.
58. Lubberstedt, M. *et al.* Serum-free culture of primary human hepatocytes in a miniaturized hollow-fibre membrane bioreactor for pharmacological in vitro studies. *Journal of tissue engineering and regenerative medicine, (Epub ahead of print, 2012)*.
59. Atkuri, K.R., Herzenberg, L.A., Niemi, A.K., Cowan, T. & Herzenberg, L.A. Importance of culturing primary lymphocytes at physiological oxygen levels. *Proceedings of the National Academy of Sciences of the United States of America* 104, 4547-4552 (2007).
60. Wang, N.X., Bazdar, D.A., Sieg, S.F. & von Recum, H.A. Microparticle delivery of Interleukin-7 to boost T-cell proliferation and survival. *Biotechnology and bioengineering* 109, 1835-1843.
61. Giese, C. *et al.* A human lymph node in vitro--challenges and progress. *Artificial organs* 30, 803-808 (2006).
62. Giese, C. *et al.* Immunological substance testing on human lymphatic micro-organoids in vitro. *Journal of biotechnology* 148, 38-45.

Erklärung / Declaration:

I, Shaikh Shimaz Hashimdeen, declare under penalty of perjury, that the present dissertation was written independently in all parts by me and the use of aids has been fully specified in this document where found necessary.

Publications of parts of this dissertation/work have been made by me as follows:

Publications made from this work:

1. Culture surface influence on T-cell phenotype and function.
   Hashimdeen SS, Römhild A, Schmück M, Kratz K, Lendlein A, Kurtz A and Reinke P.
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1. Microcirculation and Hemorheology, Dresden, Germany 2013 (Poster presentation)
   Influence of polymer scaffolds on cellular behaviour of T-lymphocytes
   Hashimdeen SS, Römhild A, Kratz K, Lendlein A, Kurtz A and Reinke P.

2. SAB retreat Berlin 2011 (Oral presentation)
   T-cell and material surface interactions
   Hashimdeen SS, Lendlein A, Kurtz A and Reinke P.

3. BCRT Immunology symposium 2009 (Poster presentation)
   Flexible closed system technologies for controlled cell expansion
   Hashimdeen SS, Klein F, Kurtz A and Reinke P.